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(54) **Silver halide emulsions with recombinant collagen suitable for photographic application and also the preparation thereof**

(57) A tabular silver halide emulsion wherein the tabular grains account for more than 75% of the total grain projected area said emulsion comprising silver halide grains nucleated in the presence of nucleation peptizer and thereafter grown in the presence of growth peptizer, wherein at least one of the peptizers is substantially pure collagen like material prepared by genetic engineering of native collagen encoding nucleic acid, said peptizer having an amino acid sequence compris-

ing more than 4 different amino acids. A process of preparing the AgX emulsion. A process of producing recombinant collagen like polypeptide comprising expression of a collagen like polypeptide encoding nucleic acid sequence by a microorganism to a degree exceeding 0.95 grammes /liter, said recombinant collagen being free of helix structure and preferably the expression occurring in a microorganism other than *E. coli* or *Saccharomyces cerevisiae*.

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**Description**

## SUMMARY OF THE INVENTION

5 [0001] The subject invention is directed at improved photographic products and improved methods of production of said products. In particular the improvement is arrived at through use of recombinant DNA technology in production of a component of a photographic product. The component of interest is collagen.

## BACKGROUND OF THE INVENTION

10 [0002] The process of photographic product making is a complex procedure about which a lot has been disclosed and patented. In general terms the process to manufacture a photographic product like a photographic paper or triacetate cellulose film consists of coating several layers on top of either a laminated paper or a transparent polymer support. These layers are known as emulsion layers which can contain the radiation sensitive silver halide crystals as the most essential component or intermediate layers without these photosensitive components. The subject invention is directed at improving the photosensitive layer as such and improving the production process of photographic layers.

[0003] There are several stages at which gelatin is used in the process of film making. The function of the gelatin is different in each stage and thus the required characteristics for each stage are different and it is to be expected that collagen like substances can be specifically tailored to suit each particular application.

20 [0004] A lot of attention has been focussed on the process of making silver halide emulsions for photographic applications. A lot of attention has been paid to the role of grain morphology of silver halide crystals and aspects that influence the AgX nucleation process and the subsequent ripening process. The most essential component in an emulsion layer of a photographic product consists of radiation sensitive silver bromide, silver chloride or silver bromochloride microcrystals optionally containing iodide which are commonly referred to as silver halide grains. A peptizer is introduced during the precipitation of the grains to avoid uncontrolled coalescence which coalescence will otherwise exhibit a number of disadvantages i.a. limit the formation of thin intermediate and high aspect ratio tabular grain emulsions which in turn is disadvantageous in photography. Gelatin in numerous forms has been used in photographic manufacturing processes as peptizer. It is well known that the tabular grains with high aspect ratio have several photographic advantages like increased sharpness, improved speed granularity relationships, increased blue and minus blue speed separation, more rapid developability and higher silver covering power (Research Disclosure Vol. 225 Jan. 1983, Item 22534; EP-A-0.610.796). It has also been desired to produce tabular grains not only with high aspect ratio but also with a narrow grain size distribution otherwise expressed as a desire for mono or homodispersity.

25 [0005] To date the gelatin used in commercial processes has been derived from animal sources in general simply by derivat from animal bone and hide. The disadvantages of this material are the presence of impurities and the fact that the nature of the composition is unclearly defined and thus not reproducible. It is unclear what components are present and in which amounts. In addition it is also unclear which components actually are required for optimal activity. The reproducibility of the photographic manufacturing process is questionable due to the lack of consistency of the gelatin composition used at various stages of the photographic manufacturing process.

30 [0006] The disadvantages of gelatin in photographic applications have been addressed in detail over the years and have been the subject of various patent applications. Most of these documents have been directed at addressing processes of developing modified gelatins after the derivat thereof from the animal source to introduce particular improvements in characteristics of the modified gelatins. In 1984 US-4.439.520 describes a desirability for more than 50% of the crystals to have aspect ratios higher than 8 as this would increase blue speed. In 1987 US patent 4.713.320 mentions using gelatin with a methionine content below 30 micromoles per gram, preferably less than 5 micromoles per gram to arrive at thin trapezoidal, hexagonal and triangular tabular grains. A normal bone derived gelatin was used which had been oxidized in order to achieve a level of methionine below 30 micromoles per gram of gelatin. The lower methionine content is also described in US 4.914.014 in 1990 as offering a wider range of pBr during precipitation conditions. Numerous publications cover processes for reducing the methionine content of gelatin. EP-558.410 published in 1993 describes oxidizing reagent reaction of alkali hypochlorite or H<sub>2</sub>O<sub>2</sub> as do articles in J.Photo.Sci 40 230-230 (Nippi), J.Photo.Sci 37, 14-18 (AGFA) of 1992 and J.Imag.Sci 33,1 3-17 of 1989. Even as early as 1959 oxidation was suggested as a manner to remove impurities.

35 [0007] There has also been a lot of research carried out on collagen and collagen like proteins per se using recombinant nucleic acid technology. The use of recombinant DNA technology in combination with collagen and the application thereof in photographic application has however been remarkably absent. Most of the documents published in the area of recombinant collagen have been directed at diagnostic applications using PCR technology on genomic nucleic acid not even requiring expression of the collagen encoding sequence. The mere presence of the sequence in the genome suffices for diagnosis in these instances. Any such documents actually mentioning expression of collagen encoding sequences certainly have not required a high degree of expression. Alternatively where expression is men-

tioned merely portions of the encoding sequence are expressed rather than the complete sequence. Often these partial sequences are used for eliciting antibodies for which amounts of proteinaceous material required are minimal. In addition once the antibodies are obtained the sequences are not required further for pharmaceutical application. Therefore in these instances low expression is not a relevant issue. Also the expression of small portions of the encoding sequence can be expected to eliminate expression problems which are attributed to the high degree of repetitiveness of the encoding sequence.

**[0008]** Synthetic nucleic acid has been designed in an effort to overcome expression problems associated with longer repetitive sequences and also in an effort to design new types of protein i.e. synthetic protein. Such synthetic polypeptides are however extremely expensive to produce. It is thus not feasible to apply such in applications requiring large scale production such as required in the field of photographic film production.

**[0009]** Most applications of the prior art thus either do not require the high degree of expression required for production on an industrial scale or do not in fact provide the desired result. The documents discussing these types of application have consequently either not addressed or have not solved the problem of obtaining high expression of native collagen sequences or sequences of corresponding length and structure.

**[0010]** In general where the prior art has suggested expressing native sequences or parts thereof general terms have been used and referrals to handbooks for general transformation protocols have been given without further detail. Any examples provided have used *E. coli* or *S. cerevisiae* as producing organism and degree of expression has been of minor importance or has not been focussed on.

**[0011]** Where expression problems of collagen like protein have been addressed this has occurred by using either modified *E. coli* or higher animal cells and insect cells. The latter are also modified for post translational processing. The application of the latter type of cells is however prohibitive for large scale production due to the high costs of the cells, the media and the isolation of product. The disadvantage of *E. coli* is that it cannot secrete the desired product. In addition the repetitiveness of a nucleic acid sequence to be expressed will provide instability of the transformed bacterium and thus result in low expression levels for any collagen like encoding sequence. It is thus not feasible to apply such production (micro)organisms in applications requiring large scale production as required in the field of photographic film production.

**[0012]** A lot of research effort has been directed at achieving the post transcription modification required to arrive at fibrillar or triple helix collagen which is the state of collagen as present in animal sources i.e. the state of collagen currently applied in industrial scale photographic applications. It has been generally accepted that host cells comprising post translational processing apparatus as such themselves or through addition of encoding sequences for post translational processing enzymes should be used when expressing collagen like material in order to arrive at collagen with a triple helix and more particularly to arrive at fibrillar collagen. It is commonly accepted that this form of collagen is the useful form for application.

**[0013]** Where the prior art has actually tentatively actually suggested recombinant collagen could be used in photographic applications the relevance of the particular form of collagen material has usually not been addressed vis a vis the requirements specific for such application. Some patent applications have mentioned in passing the use of recombinant collagen for photographic film, some even specifically mention photographic application. The teachings of such documents are clearly however directed at other issues and are not directed specifically at photographic applications and the special requirements thereof. Closer analysis actually shows that for various reasons none of the examples provided in such applications are in fact even suited for application in photographic films. The patent applications are considered non enabling and merely speculative of nature when it comes to applying recombinant collagen in photographic manufacturing processes. Examples of such documents are now provided.

**[0014]** WO90/05177 describes the production of novel polymers comprising small repeating sequences, specifically repeating groups such as silk are disclosed. Collagen is suggested as one of the structures capable of providing a repeating unit. It is stated "The properties of CLP were designed so they would undergo thermoreversible gelation at high temperatures as well as being non immunogenic. The high stability of the helices should create high tensile strength in fibres or membranes formulated from CLP. These chain properties should allow the creation of hydrogel colloids in aqueous solutions which when applied to hard substrates should act as soft coatings." A suggestion is then given of a soft coating material with a ligand for a cellular receptor. The sequence GLPGPKGDRGDAGPKGADGSP was to be added to the CLP monomer and an example of a construct to be expressed from *E. coli* is provided. With regard to this composition it is disclosed "The subject compositions may find use as wound dressings, allowing for neovascularisation, eye applications, matrices for artificial organs and the like." The combination of CLP with other repetitive functional units thereby combining functions is also suggested. However no examples are provided of sequences used.

**[0015]** The only examples provided show a recombinantly produced synthetic CLP polypeptide  $[[GAP(GPP)3]2[GPVGSP]_n$  with N-terminal and C terminal spacers. The spacers are 33 amino acids and 25 amino acids in length. Thus the repetitive GPP portion of the polymer which is 24 amino acids in length is separated by 33+25+6 amino acids. In this manner *E. coli* apparently managed to express a CLP protein of 760 amino acids, i.e. MW 63.800. The cell binding CLP had the same basic structure but the hexamer was replaced by the cell binding sequence given above

resulting in an amino acid length of 814 amino acids and a MW of 70.560. The repetitive GXY motif that is expressed is short and is separated by long none repetitive sequences. The spacer DNA encodes 2 cysteine residues and also 3 methionine residues.

[0016] The cited document states in the introductory part concerning collagen "Chemically hydrolysed natural collagen can be denatured and renatured by heating and cooling to produce gelatin which is used in photographic and medical applications among other applications. The chain property of collagen responsible for this phenomenon is its ability to spontaneously form interchain aggregates having a conformation designated as a triple helix" It is thus particularly remarked in this prior art document that helical structure was required. The subsequent text is actually silent on any photographic applications and is clearly directed at completely other matters. The subsequent text is also silent on actual degree of expression obtained by E. coli. The repetitive structure is present to such a low degree it is unlikely to retain sufficient collagen like activity to be useful in photographic application. In addition the presence of cysteine and methionine residues at the levels provided herein in the expression product in fact render such inappropriate for use in AgX emulsions for photographic applications. Furthermore it is unclear whether the use of the less repetitive sequences as described here actually provided any improved level of expression in E. coli. Thus a person skilled in the art of photographic applications would be dissuaded from applying the teaching of this document in photographic applications. Firstly because it is unclear whether industrial scale production would be feasible. Considering instability of repetitive sequences this is unlikely. Secondly it is unlikely due to the undesired presence of cysteine and methionine in AgX emulsions for photographic applications. Thirdly this is unlikely due to the absence of helical structure of the expression product. The impact thereof is totally unpredictable vis a vis stability of expression product and vis a vis applicability in photography considering the major structural difference of current gelatins.

[0017] The same inventors as the preceding cited patent application disclose in WO93/10154 high molecular weight collagen like protein polymers having repetitive triads with reduced proline content. They are stated as being capable of production in unicellular microorganisms at high molecular weights and at high efficiency. They indicate "The uniqueness of collagen repetitive tripeptide is a challenge for recombinant technology in light of the high repetitiveness of the sequence and the frequent utility of the amino acids glycine and proline in the composition. Genes encoding proteins with high levels of glycine and proline are by necessity composed of high levels of the nucleotides guanidine and cytidine in self complementary sequences. Thus as one synthesizes the gene there is substantial opportunity for strands to loop out, single stranded DNA to be excised, recombination events to occur which can result in loss of segments of the gene and inefficient transcription and/or translation. Thus there is substantial interest in developing techniques and compositions which provide the advantageous properties of collagen while at the same time allowing for stable expression of high molecular weight collagen like proteins." In addition it is stated. "The polymers will further be characterised in, being like collagen, providing helices, capable of denaturation and renaturation, forming gels etc." A molecular weight between 30-150kD is suggested and at least 45 number % of the amino acids between the glycines are proline, at least 80 weight% of triads have glycine as first amino acid, at least 40% by number of the triads comprise at least one proline. The example shows use of 3 types of repetitive GXO encoding sequences and N terminal and C terminal spacer sequences. The same spacer sequences as in the previous patent application were used. The structure of the repetitive sequences was  $[(GAHGPAAGPK)_2(GAPGPAAGPP)_{24}(GAHGPAAGPK)_2]_2 = [C]_2[A]_{24}[C]_2$ . The length of the polypeptide produced was 561 amino acids with a MW of 46.409 Dalton. In another example the repetitive sequence was  $[(GAHGPAAGPK)_2(GAPGPAAGPP)_{12}(GAHGPAAGPK)_2]_5 = [C]_2[A]_{12}[C]_2$ . The length of the polypeptide produced was 777 amino acids with a MW of 64.094 Dalton, with an observed protein band at 100kD. In the third example the structure was  $[(GAHGPAAGPK)_2(GAPGPAAGPPGSRDPGPP)_{12}(GAHGPAAGPK)_2]_4 = [C]_2[AB]_{12}[C]_2$ . The example had 1065 amino acids and MW 91,966 with a protein band visual at 135kD. Apparently smaller versions were also produced with protein band weights of 28 kD, 64 and 98 kD. With regard to expression the only details provided are that detection by western blot with antisera was carried out and that the expression of the full length polymer decreased with gene size, whilst the synthesis of full length mRNA was at equivalent levels. Another group of polymers with two other different repetitive units were produced  $[C]_2[DB]_{12}[C]_2$ ,  $[C]_2[DB]_6[C]_2$  and  $[C]_2[D]_{24}[C]_2$ , wherein B and C are as above and D=GAQGPAAGPG. Respectively these 3 proteins illustrated had 1065 amino acids and MW 91,533 D, 633 amino acids and MW 55,228 D, 1065 amino acids and MW 85,386 D with a protein band visual at 140kD. Of the examples the only information concerning characteristics of the product are provided for number 6. This product is extremely soluble in water. At room temperature or above solutions thereof of more than 8% in water are viscous but they are fluid and form to a solid gel upon chilling to 0°C. Upon heating above 28°C the gel forms a thick solution. A thermoreversible transition between liquid and gel is thus illustrated. The final example concerned a structure (GAPSQGAPGLQ)<sub>68</sub> also with the same spacers and 1077 amino acids and a MW 91.266 D. With regard to application of such polypeptide nothing more is stated than in the previously cited application of these inventors. Apparently by varying the block copolymer structure of the repetitive GXO motif it has become possible for expression of longer repetitive sequences to occur. How efficient such protein is expressed is however not clear. Yet again the expression problem due to repetitiveness is not illustrated as being solved. It is questionable industrial scale expression could be achieved. No teaching specifically concerning photographic applications is provided. In particular all examples

use spacer with cysteine and methionine which is undesirable in photographic applications. Thus a person skilled in the art of photographic applications would be dissuaded from applying the teaching of this document in photographic applications.

[0018] French patent 2685347 discusses the desirability of producing recombinant material similar in properties to gelatin. The advantage would be a more homogenous product solving reproducibility problems and the chance to modify chemical functions thereof. The idea is to produce oligopeptides as gelatin substitutes. The microorganism selected was *E. coli* and it is stated the absence of post translation modifications such as glycosylation common to *coli* is no problem. Other hosts are said to be possible. No examples are however given of such possible hosts. The nucleic acid sequence to be applied must comprise a gelatin peptide encoding sequence for (Gly-X-Y)<sub>n</sub> linked to Met-Cys-His-His-His-Leu-Met codons in order for selection to occur. The sequence given by way of example encodes Gly-Pro-Ala-Gly-Glu-Arg-Gly-Pro-Lys-Gly-Trp-Met. In a later thesis by the inventor it became apparent that the degree of expression was in fact found to be inadequate for any kind of industrial application. In addition the retrieval process of the produced amino acid was complicated. *E. coli* was the host cell described and obviously presented as a matter of fact the disadvantages already presented above for a person skilled in the art interested in industrial scale production i.e. lack of secretion, instability of repetitive sequences and thus a low degree of expression. Finally nothing was actually illustrated concerning application of the suggested collagen in a photographic film in this prior art document. Thus a person skilled in the art of photographic applications would be dissuaded from applying the teaching of this document in photographic applications due to unpredictable outcome of such structurally different material.

[0019] Finally a US patent US 5,580,712 issued to Eastman-Kodak in 1996 concerned with specifically modified collagen like polypeptides and application thereof for photographic purposes describes that collagen like peptizers with silver binding strengths below 50 mV can lead to a high degree of thin tabular grain. The document illustrates this for a number of synthetically produced polypeptides with a length of 25 amino acids. The document also mentions one polypeptide with a collagen like structure was produced using recombinant technology. The recombinant polypeptide is a synthetic polypeptide of block copolymer structure consisting of merely 4 different amino acids. No actual expression details are provided for this recombinant polypeptide, a reference is merely made to standard molecular biology production protocols and the use of *Saccharomyces cerevisiae* as expression host. The molecular weight is approximately 26kDa. It thus is questionable whether a molecular biologist familiar with the expression problems provided in detail in other documents of the same date and later would seriously contemplate such production. In addition no details concerning binding strength of the recombinantly produced product are provided thus it is also questionable whether a person skilled in the art of photographic applications would seriously consider use of this product in a silver halide emulsion for photographic product or would seriously expect it to exhibit the characteristics of the short synthetic polypeptides disclosed. The document also suggests that the specific polypeptides disclosed comprising histidine and methionine at specific points namely at Xaa of the following formula will exhibit high binding strengths and will exhibit non tabular grain formation. The formula of the compound is

Gly Pro Xaa1 Gly Leu Xaa2 Gly Pro Arg Gly Pro Pro Gly Ala Ser Gly Ala Pro Gly Phe Gln Gly. Analysis of the Table providing details of the compounds researched by Eastman-Kodak reveals that all compounds with high binding strengths had at least one reducing amino acid (=histidine or methionine) per 25 amino acids thus resulting in contents higher than 400 micromoles of methionine per gram of polypeptide. Such compounds will not be useful in nucleation/growth AgX-emulsion processes for photographic applications. The synthetic compounds illustrated as having low binding strength and favouring tabular grain formation did not comprise any reducing amino acids Met or His. A number of other US patents are issued to Eastman-Kodak on related subject matter. These patents (US 5,580,712 and US 5,670,616) revealed other synthetic fragments purported to be useful for tabular grain formation but the same single recombinant product example is described so these patents offer nothing new vis a vis recombinant collagen expression and application of recombinant collagen in photographic applications with AgE-emulsions.

It is also derivable from these descriptions of Eastman-Kodak that expression of the specific sequence shown which occurred in the host *S. cerevisiae* was in fact very low. Using 20 litres of culture merely ca 600 mgr of product could be isolated. No attention is paid to this aspect in the descriptions however. In fact the information derivable from these descriptions would dissuade the skilled person from using this system for producing collagen. As addressed already above this low degree of expression could be due to the repetitiveness of the sequence to be transcribed/translated and/or the presence of protease. In particular due to the open structure of non helical collagen any non helical collagen expression product is likely to be extremely susceptible to protease attack.

#### DESCRIPTION OF THE INVENTION

[0020] The subject invention is directed at use of recombinant DNA technology thus now finally enabling production of large amounts of substantially pure collagen material overcoming the above mentioned difficulties. For the first time a recombinant collagen production process has been executed providing high level of expression without requiring expensive media, expression hosts or non secreting expression hosts. In addition it has now become possible to

produce collagen selected and/or adapted for optimal use in each particular stage of the production process of the photographic product where gelatin has to date been applied. Thereby rendering even further improvement possible. Also an improved AgX emulsion production process is now possible leading to a reduction in production costs.

**[0021]** The subject inventors were interested in obtaining a more uniform photographic AgX-emulsion material and decided to investigate the possibility of producing native collagen identical to that of collagen derived from animal sources currently used in the industrial photographic paper and film making process. They expected that the use of recombinantly produced collagen could lead to improvements in the silver halide emulsion production due to the more uniform nature vis a vis the natural source which comprises a large number of contaminants and a mixture of collagen types of non defined and variable nature with collagen type I as major component. The idea was to use a substance coming as close as possible to the natural collagen rather than use of synthetically designed collagen like polypeptides with block copolymer structure. The hope was that expression problems for a sequence corresponding to the native sequence would be smaller than those encountered in the synthetically designed collagen like polypeptides as described in detail in the prior art. This could be hoped for on the basis of a lower repetitivity of the sequence to be expressed. On the other hand use of a more random sequence could also lead to more susceptibility to attack by various proteases.

**[0022]** However differently to the native situation for collagen we decided however to abandon attempts to produce helix like structures using the recombinant technology. In light of page 1 of WO93/07889 which states "Unless an appropriate number of  $\gamma$ -position prolyl residues are hydroxylated to 4-hydroxyproline by prolyl 4 hydroxylase the newly synthesized chains cannot fold into a triple helical conformation at 37°C. If the hydroxylation does not occur the polypeptides remain non helical, are poorly secreted by the cells and cannot self assemble into collagen fibrils" it could have been expected problems would arise upon applying secretory cells to ensure secretion. Also the potential for protease attack would be markedly higher due to the resulting open non folded structure. Thus it is surprising that our recombinant sequences result in expression products that are quite readily secreted by the expression host and in high amounts. Also the production of recombinant collagen under such conditions that the recombinant collagen compound cannot form the fibrillar structure or the triple helix structure characteristic for the native collagen that is currently used in photographic applications could also have had a questionable effect on the photographic application itself. In view of this difference with the current commercial product it was of course also questionable whether the resulting recombinant compound would be as suitable for photographic application as the helix comprising compound currently in use.

**[0023]** The results after a lot of hard work in cloning a genomic sequence for collagen types I and III, overcoming expression problems in order to produce sufficient amounts to start testing it in photographic applications were however in spite of all possible setbacks unexpectedly good. Firstly the rate of expression was unexpectedly high it was higher than 0,95 grammes/liter and was in fact higher than 3 grammes/liter overall. This is in marked contrast to the prior art where any attempts to express collagen or collagenlike material failed to produce more than milligrammes per litre if any. Thus this amazingly high rate of expression was considered totally unexpected firstly in view of all problems described in the prior art. The rate of expression however even actually outnumbered production rates achieved with expression host *Pichia pastoris* for other proteins. Quite specifically even those proteins that were not expected to be as difficult to produce in high amounts like collagen. At long last it now seems feasible a form of recombinant collagen could be produced in an economically interesting amount with an economically interesting expression host. Other high expressing hosts are to be found among microorganisms of the fungal type. In particular high expression yeasts and most specifically protease negative strains with low proteolytic activity are preferred. Yeasts that can be suitably used are quite specifically methylotrophic yeasts. A particularly suitable example is the yeast *Pichia pastoris*. On the basis of the criteria established as being relevant for expression of collagen a suitable host cell capable of expression to a degree high enough and under economically feasible conditions can be found and used.

The fact that large scale production was finally made possible by the subject inventors finally also enabled actual tests in the photographic application field to be carried out. Tests for photographic application were only made possible after sufficient recombinant collagen had been produced in relatively large amounts, which is in contrast to the small amounts required in the pharmaceutical applications described in detail in the prior art. After carrying out these tests directed at photographic application it was discovered tabular grain formation was high (see Table II). For the first time the application of substantially pure collagen type III was applied in a photographic emulsion as peptizer. The results were outstanding. Quite unexpectedly however we found a degree of tabular grain formation higher than 80%. This even outdid the Eastman-Kodak polypeptide performances of polypeptides with low binding strengths and this was considered most surprising in view of the fact that a binding strength of 69,5 mV was found for this product and thus in line with the Eastman-Kodak teaching non tabular grain formation was to have been expected. Thus the theory postulated by Eastman-Kodak concerning the requirement of the binding strength being below 50 mV to get 80% tabular grain formation is overturned. Thus the pathway was opened to develop numerous peptizers with silver binding strengths higher than those stated in the prior art as being suitable for application in silver halide emulsions requiring tabular grains at a level of more than 80%. The pathway was also opened to apply other types of collagen than type III as major collagen component of peptizer in photographic emulsions. Considering the degree of homology between the

various native collagens is 40-50%, one can expect good results also from slightly manipulated native sequences. An amino acid sequence exhibiting more than 50% homology with a native collagen amino acid sequence can be expected to provide good results. Mutations of native sequences can comprise insertions, deletions and substitutions vis a vis the native sequence. The sequences useful according to the invention must however maintain a minimum degree of variability in order to prevent expression problems of their encoding nucleic acid sequences. Thus the mutations should always result in an amino acid sequence with more than 4 different amino acids. The GXY motif should not be in the form of a block copolymer and should not comprise spacer sequences between a number of GXY motifs. Preferably more than 8, even more than 9 different amino acids should be present. In the example a protein with as many as 19 different amino acids is used. There are only 20 amino acids that occur naturally. Suitably between 10-20 amino acids or 10-19 amino acids can be used (preferably however cysteine is avoided).

**[0024]** The invention thus comprises a tabular silver halide emulsion wherein the tabular grains account for more than 75% of the total grain projected area said emulsion comprising silver halide grains nucleated in the presence of nucleation peptizer and thereafter grown in the presence of growth peptizer, wherein at least one peptizer is substantially pure collagen like material prepared by genetic engineering of native collagen encoding nucleic acid, said peptizer having an amino acid sequence comprising more than 4 different amino acids. Such an emulsion can suitably comprise a peptizer with an amino acid sequence which exhibits more than 50% homology with native collagen, preferably more than 60%. Suitably the peptizer will have a size of at least 10 kDa. Sizes between 20-80 kDa are useful in photographic application as is apparent from the examples. Peptizers of ca 600 amino acids are illustrated.

The emulsion can comprise peptizer having an amino acid sequence equivalent to that occurring in nature for collagen, wherein equivalent implies amino acid identity of at least 80%, preferably at least 90%, most preferably with an amino acid sequence the same as occurs in nature.

The emulsion can comprise a peptizer with an amino acid sequence substantially the same as occurs in nature, wherein substantially implies mutation of less than 5 amino acids, preferably less than 3. Suitable types of collagen are I, II and III. A preference exists for sequences as close to the native sequence as possible in order to assure activity and avoid expression problems. A collagen type III amino acid sequence according to the invention suitably comprises or has the sequence of figure 3. A collagen type I amino acid sequence according to the invention comprises or has the sequence of figure 8, 10 or 12. Collagen type III has the amino acid sequence defined in reference 5 of example 1 and is incorporated by reference.

**[0025]** An emulsion according to the invention wherein the peptizer is present in substantially pure form means that the peptizer is substantially free of nucleic acid, polysaccharides and other protein. The examples illustrate that this is indeed feasible. The presence of sugars and nucleic acid in even trace amounts could have some effects on crystal formation and it was indeed to be questioned whether sufficiently pure recombinant material for the specific photographic application could be produced.

**[0026]** It is advantageous when using *Pichia pastoris* as expression host to use a nucleic acid sequence encoding an amino acid sequence free of the sequence MGPR even though it is present in the native sequence of collagen type I because we have unexpectedly found this sequence is a new recognition sequence of a protease present in *Pichia pastoris* to which some collagen types are susceptible. It is postulated the protease is a Kex-2 like protease and that a Kex-2 like protease negative host strain will be a suitable host cell. In general terms when using *Pichia pastoris* as host cell it could be advantageous to use a nucleic acid sequence encoding collagen of which the corresponding amino acid sequence is free of [Leu-Ile-Val-Met]-Xaa-Yaa-Arg wherein Xaa and Yaa correspond to Gly and Pro or other amino acids and at least one of the amino acids between the brackets is amended. As the open structure of non helical collagen is susceptible to proteolysis the host should be selected and/or the sequence to be expressed is preferably mutated or selected such that proteolysis for the specific combination of host and sequence to be expressed is minimised. There are numerous options open to the skilled person to realise this on the basis of common general knowledge and the subject disclosure including the content of the cited references. Another way to increase the expression could lie in optimised codon usage for the host cell in which the sequence is introduced for expression. The use of multicopy transformants is also a way in which increased expression can be achieved. It is suggested in the art for *Saccharomyces cerevisiae* expression of bovine pancreatic trypsin inhibitor that the maximum level of protein secretion is ultimately determined by the protein folding capacity of the endoplasmic reticulum. Exceeding this capacity by the use of multicopy transformants is thought to result in the accumulation of unfolded proteins in the endoplasmic reticulum and a concomitant vast decrease in the level of expression due to physiological instability. This is described by Parekh et al in 1995 (Protein Expr. Purif. 6, 537-545) and by Parekh and Wittrup in 1997 (Biotechnol. Prog. 13, 117-122). It is feasible that this negative aspect is negated in the case of our recombinant collagen being expressed as an unfolded molecule and/or that this phenomenon is less relevant in other expression hosts in particular other yeast hosts. In yeast hosts and in bacterial hosts prolylhydroxylating mechanisms are absent and as such expression of collagen in such hosts will lead to unfolded collagen. If collagen is unfolded it will not drain the folding capacity of the endoplasmic reticulum. Also due to outstanding solubility unfolded unhydroxylated collagen will most likely not aggregate and accumulate in the endoplasmic reticulum. In order to eliminate the risk of such folding becoming relevant to degree of expression it

is thus preferred the collagen is not hydroxylated or is at least hydroxylated to as low a degree as possible.

**[0027]** It has recently been shown that it is possible to achieve hydroxylation in yeast cells such as *Saccharomyces cerevisiae* and *Pichia pastoris* by coexpression of heterologous prolyl-4-hydroxylase. This is described by Vuorela et al in 1997 (EMBO J. 16, 6702-6712) and by Vaughan et al in 1998 (DNA cell Biol. 17, 511-518). As the gelling temperature of gelatin will depend on the degree of hydroxylation it could now be possible to vary the degree of hydroxylation in a manner that will result in an expression product with a different gelling temperature. This could be of particular interest in processes with specific temperature requirements that previously prohibited economical use of collagen e. g. in processes requiring temperature above room temperature to prevent undesirable gelling.

**[0028]** The peptizer does not have to be identical to the native sequence it can be a fragment of defined length and composition derived from a native collagen encoding sequence, said fragment comprising the GXY motif characteristic of collagen, said length being such that the fragment weight on amino acid basis is at least 2,5kDa. Suitably weights can be between 2.5 and 100 kDa. Fragments of various sizes are suitable. 5-50 kDa even 20-50 kDa are suitable embodiments to be applied.

**[0029]** Various ways of ensuring absence of helix structure are available. For instance ensure the peptizer is free of hydroxyproline and/or free of procollagen and telopeptides. Preferably for photographic applications the peptizer should be free of cysteine. An AgX emulsion according to the invention, wherein the peptizer is not deaminated is an interesting further embodiment of the invention as is a peptizer with an isoelectric point of 7-10.

**[0030]** Further research was carried out to ascertain what else could be discovered to define what other parameters could be used to enhance the results. In order to do this we analysed a number of modified collagens i.e. non recombinantly produced collagens with our recombinant collagens in tests to determine relevant parameters. We subsequently defined various categories of compounds as suitable for producing silver halide emulsions with the required degree of tabular grain formation.

**[0031]** An emulsion according to the invention in any of the other embodiments already mentioned with the peptizer further comprising oxidated reducing amino acids i.e. to a degree that reducing amino acids are present at a level equivalent to a reducing strength of between 0,1-200 micromoles of methionine per gram of said peptizer is a suitable embodiment. Preferably less than 160 more preferably less than 120 micromoles of methionine per gram of said peptizer is present. A lower level of reducing power is preferred so preferred emulsions according to the invention will comprise peptizer comprising oxidated reducing amino acids to a degree that reducing amino acids are present at a level equivalent to a reducing strength of between 0,1-80 micromoles of methionine per gram of said peptizer. Quite high levels of oxidated reducing amino acids to a degree that reducing amino acids are present at a level equivalent to a reducing strength of between 30-80 micromoles of methionine per gram of said peptizer are also able to provide adequate results. This is quite surprising considering the previous teachings concerning requirements for low values of reducing power for tabular grain formation of numerous prior art publications. The invention also covers such modified collagen with lower levels of methionine than 80  $\mu$ moles e.g. modified type I. The modification e.g. to the type I collagen does not necessarily have to be by oxidation but can also be the result of mutation of the encoding sequence such that reducing amino acids are replaced to the required degree by non reducing amino acids. This means a chemical treatment step of the collagen prior to use in silver halide emulsion can be omitted with the concomitant advantages in time and cost to the production process. Obviously one can apply a native collagen which does not have more than 80  $\mu$ moles of reducing amino acid e.g. collagen type III.

**[0032]** Additionally we discovered contradictory to the teaching of Eastman-Kodak mentioned earlier and published in 1996 that an emulsion according to the invention in any of the other embodiments mentioned, comprising a peptizer with a binding strength for silver higher than 50 mV can function exceedingly well as an emulsion having a high level of tabular grain formation. A suitable peptizer will have a binding strength for silver below 100 mV. Contrary to the prior art teaching the peptizer can have a binding strength for silver between 50-100 mV and provide an emulsion with excellent tabular grain percentage.

**[0033]** The silver halide emulsion resulting from application of such collagen like material will suitably exhibit more than 80% tabular grains, preferably more than 90%. Most preferred is a tabular grain percentage higher than 95%. The grains will exhibit an average aspect ratio higher than 5 when determined using the single jet method under the reaction conditions described in the example. Note these reaction conditions are not the optimised reaction conditions used in actual photographic emulsion processes for obtaining the highest aspect ratios but merely provide an indication of whether the material is suitable to achieve high aspect ratios when such optimised conditions are applied. The compounds according to the invention upon application of optimised conditions currently used on normal collagen e. g. using the double jet method and an additional ripening process are expected to exhibit much higher aspect ratios. The applied test is merely a quick indicator of high aspect ratio forming capacity and the person skilled in the art will realise what measures can be taken to enhance the result further.

**[0034]** In our test the ripening process is carried out without any further addition of peptizer or extra silver. Obviously in a process according to the invention the ripening step could comprise such further addition. The peptizer can be the same material for both nucleation and ripening stage. An additional addition in the ripening stage could be advanta-



geous due to increased steric stability at this stage.

**[0035]** A preferred AgX emulsion according to the invention comprises a peptizer that is stable vis a vis silver halide tabular grain formation at a pH between 4-8. Conventional gelatin derived from lime bone and hydrolysed gelatin do not exhibit such good tabular grain formation results at pH higher than 5,5. The peptizers according to the invention e.g. native recombinant collagen III do exhibit such characteristic thus making the requirement for strict pH control during emulsion production storage and application redundant. The native recombinant collagen can also undergo oxidation of methionine to exhibit improved behavior. Suitably the methionine level will be less than 80 micromoles per gramme. An emulsion according to the invention can thus have a nucleation and growth pH between 4-8 without negatively affecting tabular grain formation. In the process of emulsion production a variation in pH will not negatively effect the outcome upon further processing of the resulting emulsion for photographic element production.

**[0036]** An emulsion according to the invention offers the advantage over conventional collagen comprising silver halide emulsions that the peptizer is of a homo disperse nature. The crystallisation process by virtue of this fact must also be more homogenous with all the advantages mentioned above. It is possible to add at various stages of the nucleation and growth of the silver halide crystals a further homogenous peptizer also clearly defined and substantially pure thus combining price effectiveness and controlling crystallisation properties in a regulated manner previously not possible with a collagen like peptizer. As there are many types of collagen naturally these can also be applied in photographic silver halide emulsions according to the invention.

**[0037]** There are 23 collagen genes that have been discovered so far. Most of these have been sequenced in part or as a whole. The databanks comprise the various sequences therefore. Genbank for example has the coll sequence under accession number Genbank U08020 and the collIII sequence is given in reference 5 of example 1. These native collagen genes exhibit homologies when compared among themselves of 40-50%. The relevant information of the cited references with regard to sequence data is hereby incorporated by reference see also e.g. W095/31473 page 5. Application of any of these native sequences as such or modified in order to produce a collagen like compound as defined above according to the invention, said compound subsequently being used in a silver halide emulsion for photographic application is covered by the subject invention. Suitably the native sequences are applied i.e. sequences encoding polypeptides with the natural amino acid sequences as long as the encoded polypeptide falls within the parameters disclosed above. The type I has been the type mostly applied in silver halide emulsions as the source for silver halide emulsion gelatin was animal bones of which type I collagen is the most predominantly present collagen type. Now it has become possible to also test and apply other collagen types for suitability in silver halide emulsions. The other collagen types have not been applied as such in the prior art in photographic applications and certainly not as such in silver halide emulsions. Naturally a number of them have been present in animal tissue that has been used to date. Now it has become possible to see whether these collagen types can in fact be responsible for or contribute to even more favorable properties to photographic products previously unrecognised. A photographic sensitive emulsion comprising recombinant collagen like polypeptide other than type I as collagen like component is also considered to form a suitable embodiment of the invention as long as the specific requirements set out above are fulfilled. Specifically application in a silver halide emulsion is covered. In the case of silver halide emulsions a 100% uniform source of collagen is expected to provide maximum homo dispersity of crystal formation. The requirement of homodispersity and the value thereof have been addressed elsewhere in this description of the invention. It is not necessary for the peptizer to comprise the full length collagen it can comprise a fragment thereof. Suitably such fragment is however at least 2,5 kDa, preferably more than 10 kDa in length to ensure sufficient randomness of expression whilst maintaining collagen aspects required for the peptizer.

**[0038]** Besides the emulsions described above the invention is also concerned with a process for preparing tabular silver halide emulsion wherein the tabular grains account for more than 75% of the total grain projected area said process comprising nucleation of silver halide grains in the presence of nucleation peptizer and thereafter growing said silver halide grains in the presence of growth peptizer, wherein both peptizers are present in a defined amount and at least one peptizer is collagen like material prepared by genetic engineering of native collagen encoding nucleic acid, said peptizer having an amino acid sequence comprising more than 4 different amino acids. Such a process according to the invention can comprise addition of the peptizer in the nucleation step and/or during the grain growing step, said peptizer can be selected from any of the embodiments disclosed above or in the claims. In a special embodiment the process comprises addition of the peptizer both in the nucleation step and during the grain growing step. The peptizer to be used when both steps are taken can be the same or different depending on the circumstances of the case.

**[0039]** After preparing an AgX emulsion according to the invention the AgX emulsion can undergo the standard procedures for preparing a photographic element. The emulsion can be applied in a manner known per se for achieving a silver halide emulsion layer on photographic material wherein the silver halide crystals of said layer have an aspect ratio of 5 or more.

Said photographic element is suitably a material sensitive to light, laser or x-ray radiation, said element being selected from black and white reversal film, black and white negative film, colour negative film, colour reversal film, film in which the sensitive photographic components are digitally scanned, black and white reversal paper, black and white paper,

colour paper, reversal colour paper, paper in which the sensitive photographic components are sensitized by laser radiation out of a digital database. A photographic element obtained according to such a process is also covered by the invention as well as an element using the direct positive process with internal sensitised silver halide emulsion and elements using heat development.

5 **[0040]** Another aspect of the invention lies in a process of producing recombinant collagen like polypeptide comprising expression of a collagen like polypeptide encoding nucleic acid sequence by a microorganism to a degree exceeding 0.95 grammes/liter, said recombinant collagen being free of helix structure. Preferably the expression occurs in a microorganism other than *E. coli* or *Saccharomyces cerevisiae* in order to ensure high expression and preferably secretion. The process can suitably be carried out with a fungal cell preferably a yeast cell. Suitably the host cell is  
10 selected from the group consisting of high expression host cells like *Hansenula*, *Trichoderma*, *Aspergillus* and *Pichia*. Fungal and particularly yeast cells are preferred to bacteria as they are less susceptible to bad expression of repetitive sequences. Most preferably the host will not have a high level of proteases that attack the collagen structure expressed. In this respect *Pichia* offers an example of a very suitable expression system. Preferably the microorganism is free of active post translational processing mechanism for processing collagen like sequences to fibrils thereby ensuring absence of helix structure in the expression product. Also such a process can occur when the microorganism is free of  
15 active post translational processing mechanism for processing collagen like sequences to triple helices and/or when the nucleic acid sequence to be expressed is free of procollagen and telopeptide encoding sequences. The host to be used does not require the presence of a gene for expression of prolyl-4-hydroxylase the enzyme required for collagen triple helix assembly contrary to previous suggestions in the art concerning collagen production. The selection of a  
20 suitable host cell from known industrial enzyme producing fungal host cells specifically yeast cells on the basis of the required parameters described herein rendering the host cell suitable for expression of recombinant collagen according to the invention suitable for photographic applications in combination with knowledge regarding the host cells and the sequence to be expressed will be possible by a person skilled in the art.

**[0041]** To ensure production of a non cleaved sequence a process according to the invention for producing recombinant collagen like material comprises use of a nucleic acid sequence encoding recombinant collagen amino acid sequence substantially free of protease cleavage sites of protease active in the expression host cell. In the case of *Pichia pastoris* for example and possibly also for other host cells a nucleic acid sequence encoding collagen of which the corresponding amino acid sequence is free of [Leu-Ile-Val-Met]-Xaa-Yaa-Arg wherein Xaa and Yaa correspond to Gly and Pro or other amino acids and at least one of the amino acids between the brackets is amended could be  
30 preferred. A preferred process according to the invention comprises use of the microorganism *Pichia pastoris* as expression host.

**[0042]** The process suitably provides expression leading to peptide harvest exceeding 3 grammes /liter. The process can suitably be carried out with any of the recombinant collagen like peptizers defined above for the emulsion according to the invention. As is apparent from the examples under the circumstances described therein multicopy transformants  
35 provide more than 14 grams of gelatin per liter of clarified broth at a biomass wet weight of 435 grams per liter. Most suitably the product resulting from microbial expression is isolated and purified until it is substantially free of other protein, polysaccharides and nucleic acid. As is apparent from the examples numerous methods are available to the person skilled in the art to achieve this. The process according to the invention can provide the expression product isolated and purified to at least the following degree: content nucleic acid less than 100 ppm, content polysaccharides less than 5%, content other protein less than in commercial products. More preferably the DNA content of less than 1  
40 ppm, RNA content less than 10 ppm even less than 5 ppm and polysaccharide content less than 1% can be achieved.

**[0043]** Another aspect of the invention covers novel recombinant collagen like peptides. In particular the invention covers a substantially pure collagen like material prepared by genetic engineering of native collagen encoding nucleic acid, said peptizer having an amino acid sequence exhibiting more than 40% homology with native collagen and comprising more than 4 different amino acid types. Other suitable embodiments are those peptizers described in the emulsion embodiments according to the invention. As close a homology as possible is preferred as homologies higher than 50% preferably even in the order of 80-100% are desired. We specifically point out the products are preferably free of blockcopolymer structure within the GXY motif sequence.

**[0044]** In a preferred embodiment of the invention the collagen like material comprises no cysteine residues. The presence of cysteine in photographic product will disturb the product manufacturing process. It is thus preferred that cysteine is present in as small an amount as possible. This can be achieved either through chemical modification of the recombinant product or mutation in the nucleic acid sequence encoding the product by mutation or deletion of a cysteine encoding sequence such that cysteine is no longer encoded. Suitably photographic applications will employ material comprising less than 0,1% cysteine.

55 **[0045]** In particular for the optimal silver halide emulsion homogeneity of the collagen material is of the utmost importance. It is not merely a question of absence of impurities that provides an improvement it is the possibility of providing molecules of exactly the same composition and length allowing good control of the extremely sensitive process of crystallisation and also enabling uniform crystal growth. For this reason recombinant collagen like material will

be valuable for this part of the photographic manufacturing process. In addition the absence of fibril formation and even of triple helices is required for this particular application in the photographic manufacturing process an aspect that until now had been completely overlooked. The insight in the relevance of the number of reducing groups in the photographic material is also of great importance. This is not the rigid low amount suggested in the prior art required for tabular grain formation. Thus the reduction in cysteine, histidine and methionine levels in the collagen like material to be applied forms a preferred embodiment of the invention.

[0046] The compounds according to the invention have also revealed an additional advantage. The known collagen materials e.g. regular and hydrolyzed collagen from animal sources such as bone and hide result in low tabular grain formation of the photographic film emulsion at higher pH than 5.5. The new group of recombinant collagens have been found to result in the same astonishingly high degree of tabular grain formation not only at pH 5.5 but also at higher pH e.g. pH 7. This offers the possibility of preparing silver halide emulsions which have less stringently controlled pH as the new compounds are apparently less pH dependent than the non recombinant collagens. Thus the invention is also directed at recombinant collagen like compounds that can be used in the production of silver halide emulsions at a pH between 4-8 whilst still arriving at high tabular grain percentages i.e. higher than 50% preferably higher than 80%. An additional characteristic of the recombinant collagens that can be considered useful is the fact that the isoelectric points are basic as opposed to the recombinant Eastman-Kodak polypeptide described in 1996 which has an acidic IEP. It is expected that the fact that the recombinant collagen according to the invention has an amino acid composition wherein more than 4 amino acids are present offers increased variability in the encoding sequence and thus allows higher degree of expression. Additional variety is introduced by use of a sequence with a GXY motif with less than 33% proline in the total GXY sequence. The good expression is achieved without use of a block copolymer amino acid structure in the GXY sequence.

The invention will further be illustrated by the examples.

## EXAMPLES

### Example 1

[0047] Gelatins, collagen or collagen fragments expressed as recombinant, heterologous protein in expression host organisms like yeast, fungi bacteria for photographic applications by recombinant-DNA techniques has several advantages. (i) In contrast to for example traditional gelatins, recombinant molecules can be produced as rigorously non cross-linked. (ii) The molecular composition is precisely-defined. (iii) The molecules produced are of a single type (or a well-defined mixture of only a few molecules), with minor or negligible contamination from other proteinaceous or non-proteinaceous molecules. The molecular weight distribution is very narrow and monodisperse (single-component gelatins) or oligo-disperse. (iv) The product can be manufactured in a highly reproducible way, i.e. with constant quality. Especially yeast are well-suited production organisms for such polypeptides with a highly repetitive, glycine- and proline-rich sequence.

[0048] Whereas these molecular features often cause genetic instability (e.g. recombination and shuffling of parts of the gene) in bacterial systems, this appeared to be not much of a problem in yeast [1,2]. They are eukaryotic cells, in which post translational modifications like hydroxylation can be effectuated, and which allow to choose for either efficient secretion or intracellular expression. Several species grow efficiently on cheap substrates like methanol, in contrast to animal cell cultures. Secreted production allows efficient recovery of the product during or after fermentation (contrast with plants). Several strong and tightly-regulated inducible promoters are available for yeast systems, allowing a highly efficient expression and minimizing possible negative effects on the viability and growth of the host cells. As one of several well-suited systems that are available, we have chosen for secreted production by the methylotrophic yeast *Pichia pastoris*. Our expression levels are among the highest ever reported for recombinant proteins, indicating the ability of this expression host to cope with the aforementioned of the structure of gelatin / collagen at genetic (DNA, RNA) and protein levels. After transformation of the host, the integrative is incorporated into the yeast's genome, resulting in genetical stability of the transformants (loss of plasmids is then of no importance). It is possible to generate transformants (with the heterologous target gene under the control of e.g. the alcohol oxidase (AOX) promotor), in which the recombinant gene is either incorporated into the *HIS4* locus or the *AOX1* locus. In the latter case, depending on the type of integration, the *AOX1*-gene is disrupted, leading to slow utilisation of (and slow growth on) methanol (*Mut<sup>S</sup>* phenotype). If the functional *AOX1* gene is still present, the phenotype is *Mut<sup>+</sup>*. Although both phenotypes can be used, we generally preferred fast growth and thus, our protocols were mainly directed at the generation and selection of *Mut<sup>+</sup>* transformants. It is self-evident that yeast or fungal expression systems other than the *P. pastoris* expression system could in principle be used equally well for the efficient production of recombinant gelatins, depending on the exact type and quality of molecule to be produced, on the production scale envisaged, and on the production costs and applicable market prices. The *Pichia* system was used as a fast and efficient system for pilot production and relatively easy product recovery.

## MATERIALS, METHODS & ANALYSES

### General molecular-biological techniques

[0049] Cloning procedures were carried out essentially according to Maniatis *et al.* [3]. DNA was isolated using Wizard Plus SV miniprep, or Qiagen midiprep systems. DNA was isolated from agarose gels using the QIAquick Gel Extraction Kit (Qiagen). All enzymes used were from Pharmacia unless otherwise stated and were used according to the manufacturer's recommendations. Transformation of *E. coli* was performed by standard electroporation using the BioRad GenePulser. All procedures involving the handling and transformation of *Pichia pastoris* and the expression of proteins in this host organism were essentially carried out according to the manual of the *Pichia* Expression Kit (from Invitrogen) [4].

### Insertion of a Rat COL3A1 cDNA fragment into a yeast (*Pichia pastoris*) expression vector

[0050] Plasmid pRGR5, containing a partial rat pro $\alpha$ 1(III) collagen cDNA, was a kind gift of Dr. Vuorio [5]. It was digested with PstI, yielding an approximately 0.7kb fragment of the helical domain. Using the 3'-5' exonuclease activity of T4 DNA polymerase the fragment was blunt-ended and subsequently ligated with T4 DNA ligase to SnaBI digested and CIP dephosphorylated pPIC9 *Pichia pastoris* expression vector (Invitrogen). The ligation reaction was then used to transform *E. coli* JM109.

[0051] It will be understood that the choice of possible and suitable vectors is not restricted to pPIC9. Anyone skilled in the art will be able to use and adapt a number of other possible vectors such as pHIL-S1, in which a *Pichia pastoris* acid phosphatase 1 (PhoI)-signal instead of the *Saccharomyces cerevisiae*-derived alpha-mating factor ( $\alpha$ MF) prepro signal is used, or pHIL-D1, for intracellular expression, and many others.

[0052] Plasmid DNA was isolated, and the sequence of the pCOL3A1 construct thus created (Fig. 1) was verified by sequencing according to the method of Sanger [6], using an automated sequencer (ALF DNA Sequencer, Pharmacia) and by using the 5'AOX1, 3'AOX1 and  $\alpha$ -Factor ( $\alpha$ MF) sequencing primers suggested in the *Pichia* Expression Kit (see Fig. 2). The protein sequence expected for the expressed protein is indicated in Fig. 3.

### Transformation of *Pichia pastoris* with pCOL3A1

[0053] In order to obtain Mut<sup>+</sup> transformants upon transformation of *Pichia pastoris*, the construct was linearized with Sall. In order to obtain Mut<sup>S</sup> transformants the construct was digested with BglII. After phenol extraction and ethanol precipitation, the construct was then used to transform *Pichia pastoris* strain GS115 (Invitrogen) using electroporation according to Becker and Guarente [7] using the BioRad GenePulser (set at 1500V, 25 $\mu$ F and 200 $\Omega$  and using 0.2cm cuvettes). The transformation mix was plated out on Minimal Dextrose plates (MD-plates; 1.34% YNB, 4x10<sup>-5</sup>% biotin, 1% dextrose and 1.5% agar) in order to select for the presence of the vector which converts the His<sup>-</sup> strain GS115 to His<sup>+</sup>. After growth at 30 °C for 3 days, several colonies were selected for PCR confirmation of the Mut genotype. Genomic DNA was isolated according to the yeast miniprep method of Lee [8] and RNase A treated. PCR was performed using 100ng of genomic DNA, 50pmol 5'AOX1 primer, 50pmol 3'AOX1 primer, 1.25U Taq polymerase (Pharmacia), 0.2mM dNTPs (Pharmacia) and 1x Taq buffer (Pharmacia) in a total volume of 50 $\mu$ l. After an initial denaturation at 94 °C for 5 minutes, 30 cycles were performed consisting of 1 minute at 94 °C, 1 minute at 57 °C and 2 minutes at 72 °C. Final extension was at 72 °C for 10 minutes. The PCR machine used was the Perkin-Elmer GeneAmp 480. Agarose gel electrophoresis should reveal a 2.2kb endogenous AOX1 band for Mut<sup>+</sup> transformants. Transformants without 2.2kb band are Mut<sup>S</sup>. Verified transformants of both the Mut<sup>+</sup> and Mut<sup>S</sup> genotype were selected for small-scale expression in 50ml conical tubes (placed at an angle and with the cap loosely attached), or in 100ml or 11 (baffled) flasks.

### Expression of COL3A1 fragment

[0054] Expression was performed essentially as described in the *Pichia* Expression Kit manual. Briefly, transformants were grown overnight in BMG (100mM potassium phosphate pH6.0, 1.34% YNB, 4x 10<sup>-5</sup>% biotin and 1% glycerol) to an OD<sub>600</sub>=2-6. Cultures were then centrifuged and resuspended in BMM (as BMG but 0.5% methanol replaced the glycerol) to an OD<sub>600</sub> of 1.0. Cells were grown for 4 days at 30 °C and 250rpm, with methanol being added to 0.5% every day.

[0055] 10 $\mu$ l of the culture supernatants was analyzed by SDS-PAGE according to Laemmli [9] in a BioRad mini-PROTEAN II system. Coomassie Brilliant Blue staining revealed several bands, the highest of which had the expected apparent length of about 29kD. It should be noted that gelatin, collagen and collagen fragments migrate according to an apparent Mw, which is about a factor 1.4 higher than the true Mw, at least partly due to the relatively low mean residue Mw [12].

[0056] In order to establish their identity, an SDS-PAGE gel loaded with acetone-fractionated COL3A1 fermentation supernatant (see below for the fractionation procedure) was blotted to an Immobilon P<sup>SM</sup> membrane (Millipore) using the Biorad Mini Trans-blot Cell. Quantitative transfer was achieved by applying 100V for one hour, using CAPS buffer (2.2g CAPS per liter of 10% MeOH, pH 11). After staining with Coomassie Brilliant Blue, the four most prominent bands were cut out and the N-terminal sequence was determined by Edman degradation. The 29kD band did not give any signal and is probably N-terminally blocked. One of the two smaller fragments was not pure enough to be sequenced. The other two smaller bands did give readable signals and are underlined in Fig. 3. It is clear that the bands are caused by some form of proteolysis, which can be explained by the fact that gelatin is a very open protein in the random coil conformation and is thus highly susceptible to proteolysis.

#### Protease activity

[0057] Degradation of the collagen was observed of collagen types I and III during fermentation at pH 5.0. Tests were carried out to further characterize this degradation. This degradation was markedly reduced when carrying out the fermentation process at a lower pH. Specifically pH 3.0 provided good results. We also researched the effect of addition of casamino acids. The addition gave protection for both types of collagen at pH 5.0. Furthermore the addition provided even better protection for collagen type I also at pH 3.0. This additional protection was not noticeable in the case of collagen type III at pH 3.0. It is presumed extracellular neutral proteases attack the collagen which is extremely vulnerable to proteolytic degradation resulting from its random coil conformation. (See below for a description of the fermentation procedure). The tests were carried out with the collagen-containing supernatants of a pCOL3A1 fermentation at pH 3.0, where degradation during fermentation was minimal. After removal of the cells by centrifugation, the pH of the supernatants was adjusted to pH 5.0. Subsequently, parallel incubations were carried out with the following additions:

- (1) fresh *Pichia pastoris* cells (washed with MilliQ)
- (2) fresh *Pichia pastoris* cells (washed with MilliQ) and glass beads: this mixture was vortexed (positive control I).
- (3) nothing added (negative control)
- (4) trypsin (5 mg/ml) (positive control II).

All samples were incubated for 96 hours at 30 °C and pH 5.0. (These were the conditions that caused degradation of the gelatin during fermentation). Finally, the incubated samples were analyzed on an SDS-PAGE gel according to Laemmli [9] in a Biorad mini-PROTEAN II system, followed by a Coomassie Brilliant Blue staining. The results were:

- (1) incubation at pH 5.0 with washed intact cells caused degradation of pCOL3A1 (originally produced at pH 3.0) into 4-5 discrete bands, probably as a result of cell-surface associated proteolytic enzyme activity;
- (2) addition of broken cells caused degradation of both collagen types into a large number of proteolytic products (positive control I);
- (3) no degradation occurred in the absence of cells at pH 5.0;
- (4) addition of trypsin caused massive degradation of the gelatin (positive control II). In different experiments, we verified that after removal of the cells at the end of the fermentations, the recombinant gelatins in the cell-free fermentation broth were stable for several days in the temperature range of 0-30 °C and the pH range of 3.0-7.0. Thus, some proteolytic degradation of gelatin occurred during fermentation, but after removal of cells, no relevant proteolytic activity remains and, no further precautions are necessary to stabilize the product. A similar stability was observed for the COL-1A1 products described below. This stability of the recombinant gelatins came as a surprise, as they are not hydroxylated (shown by analysis of amino acid composition, as described below) and, accordingly, non-helical, i.e. without any secondary structure. The total absence of secondary structure (i.e. of collagen-type helix) and of hydroxyproline was verified, respectively, by circular dichroic spectroscopy (CD) according to ref. [13] and by HPLC analysis of the amino acid composition after full hydrolysis of the peptide bonds. At 5 degrees Celsius it was ascertained that the expression product remained largely in the random coil configuration and is thus essentially non gelling. This is in accordance with the absence of helix stabilizing hydroxyprolines as confirmed by the experiments. The recombinant gelatins are thus extremely open molecules (and as such unparalleled polypeptides!) that are bound to be extremely prone to proteolytic degradation. The unexpected stability of the product in this expression host (also after secretion) greatly facilitates the downstream processing and isolation of the product from this expression system and obviates the repeated addition of expensive and instable inhibitors of proteolytic activity (e.g. para-methyl-sulfonyl fluoride (PMSF)). In addition, it opens up possibilities for minimizing gelatin degradation during high cell-density fermentation, viz. by continuously separating the product from the cells during fermentation, using simple micro filtration or dialysis against nutrient broth, and by recirculating the cells to the fermenter. Previously only TRIPLE-HELICAL collagen or folded polypeptides have been produced.

These are more resistant to proteases. Triple helical collagen is even fully resistant to trypsin, pepsin, and other well-known proteases. In contrast, the production of intact, non-hydroxylated and unfolded gelatin was thus expected to be extremely difficult.

## 5 Production in a protease-deficient strain

[0058] In order to investigate if the *pep4* proteinase A deficient strain SMD1168 (Invitrogen), is better suited for the expression of the protease sensitive gelatin sequences, this strain was also transformed with the pCOL3A1 construct. Methodology was as described above. Unfortunately there was no clear positive effect in both shake flask and fermenter  
10 expression experiments.

## Analysis of glycosylation

[0059] In order to establish if the protein is glycosylated, a PAS staining, involving the application of Schiff's reagent after oxidation by periodic acid, was performed on an SDS-PAGE gel. The gel was incubated for 1 hour in 12.5% TCA, 1 hour in 1% periodic acid/3% acetic acid, 1 hour in 15% acetic acid (replaced every 10 minutes) and 1 hour in Schiff's reagent (at 4 °C in the dark). The gel was then washed two times for 5 minutes in 0.5% sodium bisulfite and destained in 7% acetic acid. The expressed protein bands gave no signal, while there was a signal from a positive control (carboxypeptidase Y). As expected, no signal was obtained with a negative control (*E. coli* extract). It can be concluded  
20 that the expressed protein is not glycosylated.

## Northern Blotting

[0060] A northern blot of methanol grown cells was performed. RNA was isolated according to the method of Schmitt *et al.* [10]. The pCOL3A1 vector was digested with EcoRI/SphI to give a 0.5kb COL3A1 fragment. The fragment was <sup>32</sup>P random primer-labeled and hybridized to the blot, after which the blot was washed to a final stringency of 0.2xSSC at 65 °C. Autoradiography revealed a messenger of the expected length (1.3kb).  
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## *Pichia* transformants containing multiple copies of the heterologous COL3A1 gene

[0061] In order to investigate whether gelatin expression levels can be enhanced even further, the G418 multi copy selection method of Scorer *et al.* [11] has been applied. The pPIC9K vector was digested with BamHI/EcoRI and the 9.0kb band was isolated. The pCOL3A1 vector was also digested with BamHI/EcoRI and the resulting 1.0kb fragment was ligated to the 9.0kb pPIC9K band after which *E. coli* JM109 was transformed. The construct pCOL3A1K (Fig. 4) thus obtained was verified by restriction digestion.  
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[0062] *Pichia pastoris* GS115 was transformed with the pCOL3A1K vector as described before (digested with Sall in order to obtain Mut<sup>+</sup> transformants). In order to select for multicopy transformants, the his<sup>+</sup> colonies on MD-plates were pooled (approximately 6000) and subjected to secondary screening on plates containing a series of 10 different G418 (Gibco-BRL) concentrations ranging from 0.25 to 4.0 mg/ml. The cells were plated at a density of approximately 10<sup>5</sup> cells per plate. After incubation for 4 days at 30 °C several resistant colonies of each G418 concentration were transferred to fresh plates at the corresponding level of G418 to verify their resistance.  
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[0063] In order to determine the copy number of the pCOL3A1K vector in the G418 resistant transformants, a semi-quantitative dot blot was performed. Genomic DNA of the verified G418 resistant transformants was isolated according to the protocol of Lee [8] and RNase A treated. Approximately 200 ng of genomic DNA of each of 40 transformants (4 per concentration of G418) was transferred to a positively charged nylon membrane (Boehringer Mannheim) by means of a vacuum blotting device (Gibco-BRL Convertible system). As a 1-copy control a pCOL3A1 transformant which had been verified to contain only 1-copy by Southern blot was also transferred to the blot (*in duplo*), as well as a non-transformed control (*in duplo*).  
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[0064] The pCOL3A1 vector was digested with EcoRI/SphI to give a 0.5kb COL3A1 fragment. This fragment was <sup>32</sup>P random primer-labeled and hybridized to the dot blot filter. After washing to a final stringency of 0.5xSSC at 65 °C, autoradiography was performed. After stripping (the efficiency of which has been checked), the membrane was hybridized to a probe derived from a verified *Pichia pastoris* URA3-fragment, which had been picked up by PCR with heterologous URA3 primers. This control serves for normalization of the COL3A1 signals for the amount of DNA loaded. The membrane was washed and subjected to autoradiography as described for the COL-3A1 probe. The signals on both autoradiograms were densitometrically quantified using a gel scanner (PDI, Pharmacia). As expected there was  
45 no COL3A1 signal for the 0-copy controls, while there was a URA3 signal. The copy number can be estimated by calculating the ratio of COL3A1 signal for each transformant and the average COL3A1 signal obtained for the 1-copy controls, as normalized by the ratio of the respective URA3 signals (i.e. to account for differences in the amount of  
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DNA blotted to the membrane). Transformants containing approximately 1 to 15 copies were thus obtained.

#### Expression of COL3A1 fragments in multi-copy transformants.

**[0065]** A series of transformants containing 1 to 15 copies was subjected to small-scale expression as described above. Since SDS-PAGE indicated a higher yield at higher copy number, further tests were carried out at a 100 mL scale with the 2-, 5-, 10- and 15-copy transformants. They were grown overnight in 100 ml flasks, containing 25 mL BMG (100 mM potassium phosphate pH 6.0, 1.34% YNB,  $4.10^{-5}\%$  biotin and 1% glycerol). After centrifugation at 1500-3000 g for 5 minutes, the cells were resuspended in 100 ml BMM (as BMG but 0.5% methanol instead of the glycerol).

**[0066]** They were grown in 1 liter baffled flasks at 30°C and 250 rpm for 4 days, with methanol being added to 0.5% every day. 1 ml samples were taken each day and analyzed on SDS-PAGE. A higher copy number resulted in a higher amount of gelatin product. Selected 5- and 15-copy transformants were used for expression tests in a fermenter at a 1 L-scale. The highest COL3A1 production was obtained with the 15-copy transformant (about 14.8 g gelatin / L in the extracellular medium at a dry biomass of 177 g/L and after about 184 hours of fermentation, i.e. about 7.7 g/L overall, or 42 mg/(L.hour); at a dry biomass of 110 g/L and after about 120 hours of fermentation, it was about 7 g gelatin / L in the extracellular medium, i.e. about 3.7 g/L overall, or 31 mg/(L.hour)).

#### Cloning of a mouse COL1A1 fragment (COL1A1-1) mouse

**[0067]** Primers were designed on the known sequence (Fig. 5). PCR was performed on Mouse 17-day Embryo QUICK-Clone™ (fibroblast) cDNA (Clontech), using 0.4 ng of cDNA, 0.4 μM C1A1-FW primer (Fig. 5), 0.4 μM C1A1-RV1 primer (Fig. 5), 1x Advantage KlenTaq Polymerase Mix (Clontech), 0.2 mM dNTP's (Pharmacia) and 1x KlenTaq PCR reaction buffer (Clontech) in a total volume of 20 μl. After an initial denaturation at 94°C for 4 minutes, 35 cycles were performed of 1 minute at 94°C, 1 minute at 68°C and 2 minutes at 72°C. Final extension was at 72°C for 10 minutes. Agarose gel electrophoresis shows a 1 kb band, which is the size predicted from the sequence. DNA was isolated from the agarose gel and subsequently digested with *Nco*I and *Xho*I restriction enzymes. The digested fragment was isolated from agarose gel and cloned into the *Pichia pastoris* expression vector pPIC9 according to the following strategy (Fig. 6). First, an adaptor containing a *Nco*I and a *Xho*I site, was inserted in the multiple cloning site of pPIC9, yielding pPIC9\*. The adapter was prepared by annealing the synthetic oligonucleotides N-X-FW and N-X-RV as shown in Fig. 5 and Fig. 6. The single-strand overhang originating from the 5' end of the oligonucleotide N-X-RV was designed to form an *Eco*RI site after annealing with the *Eco*RI-digested vector. The 5' overhang from N-X-FW (*Xho*I\*) was complementary to the overhang created by the action of *Xho*I on the vector, but did not give rise to an *Xho*I site after ligation. Because the target vector, pPIC9, has an *Nco*I site outside the multiple cloning site, pUC18 has been used as an intermediate vector for the cloning of this fragment. The section between *Bam*HI and *Eco*RI of the altered multiple cloning site of pPIC9\* was transferred to the multiple cloning site of pUC18 vector, resulting in pUC18\*. The *Nco*I-*Xho*I digested fragment COL-1A1-1 was ligated in the pUC18\* vector between the *Nco*I and *Xho*I sites. From this pUC18-COL1A1-1 construct the COL1A1-1 fragment, together with the part of the multiple cloning site from the pPIC9, was digested with *Bam*HI and *Eco*RI and ligated in pPIC9, yielding the construct pCOL1A1-1 (Fig. 7). Thus, a partial *Nco*I-digestion of the pPIC9 was not necessary. The correct insertion in pPIC9 was first checked by restriction analysis and then by DNA sequencing.

#### Transformation of *Pichia* with pCOL1A1-1 and expression of the COL1A1-1 fragment

**[0068]** *Pichia pastoris* GS115 was transformed with the pCOL1A1-1 vector as described for the pCOL3A1 vector. *Sal*I-digested DNA was used in order to specifically generate Mut<sup>+</sup> transformants. Several transformants were used for small-scale expression in shaking flasks and one of those was selected for expression in the fermenter at a 1-100 L scale. Typical yields are in the range of 4-5 g gelatin / L in the (extracellular) medium (as determined after acetone fractionation, described below), at a dry biomass of 100-120 g/L (about 3 g gelatin / L overall). The target gelatin (Fig. 8) has a theoretical Mw of 27.4 kD. Collagenous proteins and gelatin are known to migrate at an apparent Mw approximately 1.4 times higher than the true Mw [10]. In agreement, an SDS-PAGE band migrating at an apparent Mw of about 38 kD was observed (interpolated value obtained with globular protein Mw markers). In addition, three shorter products with an apparent Mw of 24, 18 and 15 kD were observed (interpolated values). These could be the result of early proteolytic activity in the intracellular, cell surface-associated or extracellular compartments, or from problems at the level of translation. The degradation products were present already at very early stages of induction and no further degradation occurred. Even incubation in the presence of washed intact cells at pH 5.0 and 30 °C during 96 hours did not cause further degradation of pCOL1A1-1 with respect to the situation after fermentation at pH 3.0. (Massive degradation occurred in the presence of trypsin, as a positive control). In order to verify that problems at the mRNA level

were not responsible for the occurrence of the 24, 18 and 15 kD products, Northern blotting was performed as described for COL3A1, using a  $^{32}\text{P}$  random-primer labeled 1.0kb NcoI/XhoI COL-1A1-1 fragment from pCOL1A1-1 as the probe. The expected 1.6 kb messenger was found.

[0069] In order to establish the identity of the observed fragments, an SDS-PAGE gel loaded with acetone-fractionated COL1A1-1 fermentation supernatant was blotted to an Immobilon P<sup>SO</sup> membrane (Millipore) using the Biorad Mini Trans-blot Cell. (See below for a description of the acetone fractionation procedure). Quantitative transfer was achieved by applying 100V for one hour, using CAPS buffer (2.2g CAPS per liter of 10% MeOH, pH 11). After staining with Coomassie Brilliant Blue, the four most prominent bands were cut out and the N-terminal sequence was determined by Edman degradation. The sequencing signals obtained were extremely low as compared with the amount of loaded material (on average around 5%). It is therefore likely that the fragments were for the most part N-terminally blocked. This supports the idea that proteolysis of COL1A1-1 takes place intracellularly. The large amount of COL1A1 supplied, allowed nevertheless an easy determination of the N-terminal sequence. The N-terminal sequences obtained are underlined in the protein sequence (Fig. 8) as encoded by the transfected COL1A1-1 gene. The fragments with an apparent Mw of 38 kD and 18 kD both gave the sequence expected for the N-terminus of the protein, but extended with 'EA'. This extension (or EAEA) is known to be present on some proteins expressed from expression vectors utilizing the *Saccharomyces cerevisiae*-derived alpha-mating factor ( $\alpha\text{MF}$ ) prepro signal. This effect is assumed to be due to steric hindrance of STE13 cleavage activity. However, because most of the protein is probably N-terminally blocked, it may well be that this extended version represents only the minor fraction that is sequenceable. Based on the N-terminal and internal sequences, the fragments with an apparent Mw of 38, 24, 18 and 15 kD were assigned to be fragments consisting of, respectively, residue 1-310, 126-310, 1-125, and 42-125 of the target product as shown in Fig. 8, and having theoretical Mw's of 28, 16, 12, and 8 kD, respectively. Fragments corresponding to residue 1-41 (theoretical Mw 4 kD) and 42-310 (theoretical Mw 24 kD, apparent Mw 34 kD) were not observed. This could be due to a (much) more frequent cleavage between residue 125 and 126 than between residue 41 and 42.

#### Cloning and expression of mouse COL1A1-2

[0070] PCR has been carried out on Mouse 17-day Embryo QUICK-Clone<sup>TM</sup> cDNA (Clontech) in the same way as mouse COL1A1-1, using C1A1-FW and C1A1-RV2 primer. After denaturation at 94°C for 4 minutes, 35 cycles were performed of 1 minute at 94°C, 1 minute at 65°C and 3 minutes at 72°C. Final extension was at 72°C for 10 minutes. Agarose gel electrophoresis shows a 1.8 kb band, which is the size predicted from the sequence. The further cloning of COL1A1-2 into the pPIC9 expression vector has been carried out in the same way as COL1A1-1, yielding pCOL1A1-2 (Fig. 9).

[0071] *Pichia pastoris* GS115 was transformed with the pCOL1A1-2 vector as described for the pCOL3A1 vector. *SalI*-digested DNA was used in order to specifically generate Mut<sup>+</sup> transformants. Several transformants were used for small-scale expression in shaking flasks and one of those was selected for expression in the fermenter at a 1-100 L scale. Fig. 10 shows the expected COL1A1-2 amino acid sequence. Typical yields are in the range of 4-5 g gelatin / L in the (extracellular) medium, at a dry biomass of 100-120 g/L (about 3 g gelatin / L overall). The target gelatin (Fig. 10) has a theoretical Mw of 53 kD. In agreement with this value (and with the known anomalous migration of gelatin in SDS-PAGE [10]), a SDS-PAGE band migrating at an apparent Mw of about 74 kD was observed (interpolated value obtained with globular protein Mw markers). In addition, three shorter products with an apparent Mw of 56, 18 and 15 kD were observed (interpolated values). If the proteolytic cleavage would occur at corresponding sites in the COL1A1-1 and COL1A1-2 expression products (Fig. 8, 10), fragments consisting of residue 1-595, 126-595, 1-125, and 42-125 of the COL1A1-2 product would be expected to occur. These would have theoretical Mw's of 53, 42, 12, and 8 kD, respectively, corresponding to apparent Mw's of 74, 58, 17 and 11 kD. Surprisingly, this corresponds well to the observed apparent Mw's, indicating that cleavage of COL1A1-2 was restricted mainly to the bond between residue 125 and 126, and (in addition), the bond between 41 and 42. Again, fragments corresponding to residue 1-41 (theoretical Mw 4 kD) and 42-595 (theoretical Mw 50 kD, apparent Mw 70 kD) were not observed. This could be due to a (much) more frequent cleavage between residue 125 and 126 than between residue 41 and 42, as mentioned for COL1A1-1.

#### Cloning and expression of mouse COL1A1-3

[0072] PCR has been carried out on Mouse 17-day Embryo QUICK-Clone<sup>TM</sup> cDNA (Clontech) in the same way as mouse COL1A1-1, using C1A1-FW and C1A1-RV3 primer. After denaturation at 94°C for 4 minutes, 35 cycles were performed of 1 minute at 94°C, 1 minute at 65°C and 3 minutes at 72°C. Final extension was at 72°C for 10 minutes. Agarose gel electrophoresis shows a 2.8 kb band, which is the size predicted from the sequence.

[0073] The PUC18<sup>+</sup> plasmid was digested with NcoI and dephosphorylated. The C1A1-FW/C1A1-RV3 PCR product was digested with NcoI and the resulting 2.5 kb fragment was gel purified and ligated into the NcoI digested and dephosphorylated vector. After transformation of *E. coli* XL1Blue, correct orientation of the insert in the resulting clones



was verified by PvuII digestion. The further cloning of COL1A1-3 into the pPIC9 expression vector has been carried out in the same way as described for COL1A1-1, yielding pCOL1A1-3 (Fig. 11).

[0074] *Pichia pastoris* GS115 was transformed with the pCOL1A1-3 vector as described for the pCOL3A1 vector. *SalI*-digested DNA was used in order to specifically generate Mut<sup>+</sup> transformants. Several transformants were used for small-scale expression in shaking flasks and one of those was selected for expression in the fermenter at a 1-100 L scale. Fig. 12 shows the expected COL1A1-3 amino acid sequence. Typical yields are in the range of 4-5 g gelatin / L in the extracellular compartment (determined after acetone fractionation, as described below), at a dry biomass of 100-120 g/L (about 3 g gelatin / L overall). The target gelatin (Fig. 12) has a theoretical Mw of 72 kD. In agreement with this value, an SDS-PAGE band migrating at an apparent Mw of about 100 kD was observed (interpolated value obtained with globular protein Mw markers). In addition, three shorter products with an apparent Mw of 85, 18 and 15 kD were observed (interpolated values). If the proteolytic cleavage would occur at homologous sites in the COL1A1-1 and COL1A1-3 expression products (Fig. 8, 12), fragments consisting of residue 1-812, 126-812, 1-125, and 42-125 of the COL1A1-3 product would be expected to occur. These would have theoretical Mw's of 72, 60, 12, and 8 kD, respectively, corresponding to apparent Mw's of 100, 84, 17 and 11 kD. Surprisingly, this corresponds well to the observed apparent Mw's, indicating that cleavage of COL1A1-3 was restricted mainly to the bond between residue 125 and 126, and (to a lesser extent), the bond between 41 and 42. Again, fragments corresponding to residue 1-41 (theoretical Mw 4 kD) and 42-812 (theoretical Mw 68 kD, apparent Mw 96 kD) were not observed. This could be due to a (much) more frequent cleavage between residue 125 and 126 than between residue 41 and 42, as mentioned for COL1A1-1.

[0075] Table 1 summarizes the apparent COL1A1 fragment sizes derived by comparison with molecular weight marker proteins (LMW Calibration kit; Pharmacia), together with the size calculated from the sequence.

Table 1:

Apparent and theoretical molecular weights of COL1A1 fragments				
Apparent molecular weight on SDS-PAGE gel	Molecular weight corrected for 40% slower migration	Residue	Molecular weight calculated from the sequence	Gene product
100 kD	71 kD	1-812	72 kD	(1A1-3)
85 kD	61 kD	125-812	61 kD	(1A1-3)
74 kD	53 kD	1-595	54 kD	(1A1-2)
56 kD	40 kD	125-595	42 kD	(1A1-2)
38 kD	27 kD	1-310	28 kD	(1A1-1)
24 kD	17 kD	125-310	16 kD	(1A1-1)
18 kD	13 kD	1-125	12 kD	(1A1-1,2,3)
15 kD	11 kD	42-125	8 kD	(1A1-1,2,3)

[0076] It is clear from table 1 that the 'MGPR' model fits the actual found fragment sizes well.

[0077] One would also expect fragments of residue 1-41 (4 kD; 1A1-1,2,3) and 42-310 (theoretical Mw: 24 kD apparent Mw: 34 kD; 1A1-1), 42-595 (theoretical Mw: 50 kD apparent Mw: 70 kD; 1A1-2), or, 42-595 (theoretical Mw: 68 kD apparent Mw: 96 kD; 1A1-3). The fact that these fragments are not seen on gel, may be explained by assuming that the rate of cleavage at the second 'MGPR' site is much higher than that at the first site. This means that if the protein molecule is cut, it will always first occur at the second site. This difference in cleavage rates may be explained by the fact that the first site is preceded by a proline residue which may sterically hinder the protease.

#### Mouse COL1A1-1, COL1A1-2 AND COL1A1-3 'RGPM' mutants

[0078] We considered the possibility that the same amino acid sequence, functioning as recognition site for proteolytic enzyme(s), would be responsible for the degradation of all COL1A1 products (COL1A1-1, COL1A1-2, COL1A1-3). Surprisingly, both internal N-terminal sequences obtained from the 15 kD and 24 kD COL1A1-1 fragments were preceded by the same sequence 'MGPR'. Moreover, this sequence occurs only twice in the mouse COL1A1-1, COL1A1-2, or COL1A1-3 genes, viz. at residue 83-41 and 122-125. (As compared to COL1A1-1, COL1A1-2 and COL1A1-3 do not contain additional MGPR-sites). Also, the COL3A1 fragment from the rat did not contain such a site. This corresponds nicely to the observed cleavage pattern. Therefore, we think that 'MGPR' is a motif recognized by a specific protease, resulting in the cleavage of the COL1A1 proteins. This MGPR protease recognition site has not been described previously. A more generalized representation of the motif could possibly be MXXR, MXX[RK], or even [MLIV]XX[ARKH]. The former motifs are indeed present only twice in the entire mouse COL1A1 gene and are absent in the

COL3A1 fragment, while the latter motif is so broad that it includes non-cleaved sites: MKGH at residue 85-88 and VGAK at residue 169-172 in COL1A1, and IKGH at residue 198-201 in COL3A1. Thus, MXXR or MXX[RK] are more likely generalized motifs than [MLIV]XX[RKH]. Fig. 13 shows the 'MGPR' motifs in the COL-1A1-2 sequence. It is to be expected that this proteolytic cleavage site is only recognized by the enzymes involved if it occurs in relatively open, unfolded structures (like in our gelatins), but not so easily in more compactly folded structures (like in globular proteins). Thus, it may be important only in certain classes of proteins and polypeptides, including gelatins and unfolded collagens. [0079] In order to be able to produce full-length COL1A1-1, COL1A1-2 AND COL1A1-3 without the occurrence of the three other main bands, the 'MGPR' motifs should be removed by site-directed mutagenesis. In order to maintain the original amino acid composition of natural COL1A1 gelatin, the 'MGPR' motif was removed by converting it to 'RGPM'. Two pairs of complementary primers were synthesized:

## COL1A1MUT1FW:

R G P M

5'-GAG-CCT-GGC-GGT-TCA-GGT-CCA-CGA-GGT-CCA-ATG-GGT-CCC-CCT-  
GG-3'

## COL1A1MUT1RV:

5'-CC-AGG-GGG-ACC-CAT-TGG-ACC-TCG-TGG-ACC-TGA-ACC-GCC-AGG-  
CTC-3'

## COL1A1MUT2FW:

R G P M

5'-GGA-GCT-CCT-GGC-CAG-CGA-GGT-CCA-ATG-GGT-CTG-CCC-GGT-GAG-  
AG-3'

## COL1A1MUT2RV:

5'-CT-CTC-ACC-GGG-CAG-ACC-CAT-TGG-ACC-TCG-CTG-GCC-AGG-AGC-  
TCC-3'

[0080] Note: mutant positions are underlined; the original C of the Pro residue has been converted into A to avoid the generation of an NcoI site.

Three primer combinations were used:

1. 5'AOX1 primer and COL1A1MUT1RV
2. COL1A1MUT1FW and COL1A1MUT2RV
3. COL1A1MUT2FW and 3'AOX1

[0081] The reactions contained: 1.25 U Pwo polymerase (Eurogentec), 50 pmol of each primer, 0.2 mM dNTPs (Pharmacia), 1x Pwo buffer (Eurogentec) and 15 ng pCOL-1A1-1 template DNA in a total reaction volume of 50 µl. The PCR-machine used was the GeneAmp 9700 (Perkin-Elmer). After an initial incubation at 94 °C for 5 minutes, 18 cycles

were performed consisting of 30 seconds at 94 °C, 30 seconds at 60 °C and 45 seconds at 72 °C. Final extension was performed at 72 °C for 10 minutes. Agarose electrophoresis of the PCR-reactions revealed products of the expected sizes (0.5, 0.3 and 0.7 kb respectively). The bands were cut out from the gel and purified. The isolated fragments were then subjected to overlap-extension PCR. Approximately 0.1 pmol of each fragment was mixed together. 50 pmol of 5'AOX1 and 3'AOX1 primer were added, as well as Pwo polymerase, dNTPs and buffer as described above. Cycling conditions were the same as described above with the exception that extension at 72°C was performed for 90 seconds instead of 45 seconds. Agarose gel electrophoresis revealed the expected 1.5 kb product. The remainder of the PCR reaction was purified using the QIAquick PCR Purification Kit (Qiagen). The purified DNA was then digested with BamHI/ApaI, after which the resulting 1.0kb fragment was purified from gel. *E.coli* strain JM110 was transformed with pCOL1A1-1, pCOL1A1-2 and pCOL1A1-3, in order to remove the *dcm* methylation of the ApaI site. After DNA isolation, the plasmids were digested with BamHI/ApaI. The resulting vector fragments of 7.9, 8.8 and 9.5 kb, respectively, were purified from agarose gel and ligated to the BamHI/ApaI digested PCR-product. *E. coli* XL1-Blue was transformed with these ligation reactions and plasmid DNA of PCR-verified insert containing clones was isolated and verified by automated sequencing. The mutant plasmids pCOL1A1-1\*, pCOL1A1-2\* and pCOL1A1-3\* thus created were digested with Sall and used to transform *Pichia pastoris* strain GS115. Small- and fermentor-scale expression was performed as described for COL1A1-1.

[0082] The SDS-PAGE analysis clearly shows that only one major band of the expected full-length size is formed, for COL1A1-1\* as well as COL1A1-2\* and COL1A1-3\*.

## Expression / production of gelatin in a fermentor

[0083] Fed-batch fermentations were performed according to the *Pichia* fermentation process guidelines of Invitrogen. Cells were grown in a 1-liter fermentor (Applikon) in the initial experimental stages to optimize protein production. Thereafter cells were grown in a 20-liter or a 140-liter fermentor (Biobench 20, Bio-pilot 140, Applikon) for pilot scale production of collagen. Working volumes were 1-liter, 15-liter and 100-liter, respectively. AD1020 controllers (Applikon) were used to monitor and control the fermentation parameters. The program BioXpert (Applikon) was used for data storage. Dissolved oxygen levels were monitored in the fermentor using an oxygen electrode (Ingold for 1-liter fermentations, Mettler Toledo for larger scale fermentations). Agitation (500 - 1000 rpm) and aeration (1 - 2 vvm, i.e. 1-2 LL<sup>-1</sup>min<sup>-1</sup>) were manually adjusted to keep the dissolved oxygen concentration above 20%. pH was measured by a pH electrode (Broadly James cooperation) and automatically kept at pH 3.0 or pH 5.0 by addition of ammonium hydroxide (25%), which also served as nitrogen source for growth of the microorganisms. An anti foam-electrode was used to prevent excessive foaming. When necessary, the anti foam Structol J673 (Schill and Seilacher, Hamburg, Germany) was used. Growth of the microorganisms was monitored by determination of the cell dry weight. A calibration curve was made by which cell wet weight could be converted into cell dry weight. Cell wet weight was determined after centrifugation of 2 ml-samples for 5 min at 15.000 rpm and removing the supernatant. Cell dry weight was determined after addition of 200 µl of cells to a pre-dried filter (0.45 µm membrane, Schleicher & Schüll, Dassel, Germany), washing with 25 ml of deionized water and drying e.g. in a microwave oven for 15 minutes at 1000 W. Cell dry weight was approximately a factor 3 lower than cell wet weight. Precultures were started from colonies on a MGY plate, in flasks containing a total of 10% of the initial fermentation volume of MGY. The volume of the medium was ≤20% of the total flask volume. Cells were grown at 30°C at 200 rpm in a rotary shaker (Gallenkamp) for 24-60 hours.

### Fermentation medium

[0084] The fermentation basal salts medium in the fermentor contained per liter: 26.7 ml of phosphoric acid (85%), 0.93 g calcium sulfate, 18.2 g potassium sulfate, 14.9 g magnesium sulfate.7H<sub>2</sub>O, 4.13 g potassium hydroxide and 40.0 g glycerol. An amount of 4.3 ml of PTM<sub>1</sub> trace salts was added per liter of fermentation basal salts medium. PTM<sub>1</sub> trace salts contained per liter: 4.5 g cupric chloride.2H<sub>2</sub>O, 0.09 g potassium iodide, 3.5 g manganese chloride.4H<sub>2</sub>O, 0.2 g sodium molybdate.2H<sub>2</sub>O, 0.02 g boric acid, 1.08 g cobalt sulfate.7H<sub>2</sub>O, 42.3 g zinc sulfate.7H<sub>2</sub>O, 65.0 g ferrous sulfate.7H<sub>2</sub>O, 0.2 g biotin and 5.0 ml sulfuric acid. Trace salts were filter sterilized (pore size 0.22 µm, Costar, USA). Casamino acids (caseine hydrolysate Merck) were separately sterilized and added to the fermentation medium in an amount of 5g/l when collagen type I from the mouse was expressed (COL1A1-1, COL1A1-1\*, COL1A1-2, COL1A1-2\*, COL1A1-3, COL1A1-3\*). During the fermentation after 50 hours a further amount of 5g/l of sterile casamino acids was added to the fermentation medium.

### Fermentation of mut<sup>+</sup> cultures

[0085] The fermentor was sterilized with the fermentation basal salts medium. The 20-liter and 120-liter fermentor were sterilized in situ with initial medium volumes of 5-7.5 l and 50-liter, respectively. The 1-liter fermentor was sterilized

with 500 ml medium in an autoclave. After sterilization the temperature was set at 30°C, agitation and aeration were set at 500 rpm and 1 vvm (i.e. 1 LL<sup>-1</sup>min<sup>-1</sup>), respectively. The pH was adjusted to set point (usually pH 5.0) with 25% ammonium hydroxide. Trace salts were aseptically added to the medium. The fermentor was inoculated with 10% of the initial fermentation volume of precultured cells in MGY. The batch culture was grown until the glycerol was completely consumed (18-24 hours). This was indicated by an increase of the dissolved oxygen concentration to 100%. Cell dry weight was 25-35 g/l in this stage. Thereafter the glycerol fed-batch phase was started by initiating a 50% (v/v) glycerol feed containing 12 ml PTM<sub>1</sub> trace salts per liter of glycerol. The glycerol feed was set at 18 ml/h/liter initial fermentation volume. The glycerol feed was carried out for 4 hours, or overnight in the case of a long lag phase. During the glycerol batch phase the pH of the fermentation medium was lowered to 3.0. The protein induction phase was initiated by starting a 100 % methanol feed containing 12 ml PTM<sub>1</sub> trace salts per liter of methanol. The feed rate was set to 3 ml/h/liter initial fermentor volume. During the first hours methanol accumulated in the fermentor. After 2-4 hours dissolved oxygen levels decreased due to adaptation to methanol. The methanol feed was increased to 6 ml/h/initial fermentor volume in the case of a fast dissolved oxygen spike. If the carbon source is limiting, shutting of the carbon source causes the culture to decrease its metabolic rate and the dissolved oxygen concentration rises (spike). After an additional 2 hours the methanol rate was increased to 9 ml/h/liter initial fermentor volume. This feed rate was maintained throughout the remainder of the fermentation. The fermentation was stopped after 70-130 h methanol fed-batch phase. During the fermentation samples were taken of 2 ml, centrifuged (5 min, 15.000 rpm) and the supernatant was stored at -20°C.

[0086] Concentration of gelatin and total protein was determined after filtration of the samples (0.22 µm) and subsequent acetone fractionation (40 vol-%, followed by 60-80 vol-% acetone). The BCA protein assay (Pierce) was routinely used, with gelatin from Merck as a reference. According to SDS-PAGE and analysis of the amino acid composition, the non-collagenous proteins precipitated at 40 % acetone, while the COL3A1 and COL1A1 fragments precipitated at 60-80 %. At 60 % acetone, preferentially the higher molecular weight gelatin components precipitated. At increasing acetone concentration, increasing precipitation was obtained for the main degradation products described above. At 80 %, all of the main degradation products were recovered in the precipitate (as checked with SDS-PAGE). Small peptides and other low molecular weight contaminants remained in solution at 80 % acetone.

[0087] At the end of the fermentation, the cells were removed by centrifugation (10.000 rpm, 30 min, 4°C) in the case of the 1-liter fermentation. Cells were removed by micro filtration in the case of the 20-liter fermentation. The cell broth was first applied to a micro filtration module containing a polyether sulfone membrane with 0.50 µm pore size (type MF 05 M2 from X-Flow, fitted in a RX 300 filtration module from X-Flow). Thereafter the supernatant was applied to a similar type of micro filtration module containing a polyether sulfone membrane with 0.2 µm pore size (type MF 02 M1, similarly from X-Flow). In the case of the 120-liter fermentation cells were removed by a pilot plant scale micro filtration unit containing a polyether sulfone membrane with 0.2 µm pore size (type MF 02 M1, from X-Flow, fitted into a R-10 membrane module). These filtration units are mentioned merely as examples. It will be understood that any suitable micro filtration system could be applied to remove the cells. Optionally, the bulk of cells and debris was removed by centrifugation, and only the supernatant and the medium used to wash the cells was applied to the microfiltration units. Alternatively, it is possible to recover the product from the fermentation broth and separate it from the cells by directly applying the fermentation broth to a suitable expanded bed chromatography system, using a resin that specifically binds the gelatin produced. We successfully used SP Sepharose XL Streamline from Pharmacia as a cation exchanger in expanded bed mode, at pH 3-4.

#### Fermentation of Mut<sup>s</sup> cultures

[0088] Glycerol batch and fed-batch phase were performed as described for the mut<sup>+</sup> cultures. Since Mut<sup>s</sup> cultures metabolize methanol poorly, their oxygen consumption is very low. Therefore spikes of the dissolved oxygen concentration cannot be used to evaluate the culture. The methanol feed was adjusted to maintain an excess of methanol in the medium which does not exceed 0.3%. The methanol feed was initiated at 1 ml/h/liter initial fermentor volume and increased slowly to 3 ml/h/liter. The total fermentation time required when using Mut<sup>s</sup> cultures was comparatively longer than when Mut<sup>+</sup> cultures were used.

#### **Preparative purification of collagen / gelatin on a preparative scale**

[0089] After the micro filtration step, two alternative purification strategies were followed (see I, II below).

## I. Purification by differential precipitation

Acetone fractionation

[0090] Collagen type I and type III were partly purified from batches of 500 ml to 2 liter of supernatant by a 40-80% acetone fractionation. At 40% acetone, the non-collagenous proteins (from *Pichia*) were precipitated, while at 60-80% acetone, collagen as well as collagen breakdown products were precipitated, as shown by SDS-PAGE and analysis of the amino acid composition. At 60 % acetone, preferentially the higher molecular weight gelatin components precipitated. At increasing acetone concentration, increasing precipitation was obtained for the main degradation products described above. At 80 %, all of the main degradation products were recovered in the precipitate (as checked with SDS-PAGE). Small peptides and other low molecular weight contaminants remained in solution at 80 % acetone. Acetone was cooled for 2-4 hours at -20°C. An amount of 40% of ice-cold acetone (v/v) was added slowly to the pre-cooled supernatant from the fermentation at 4°C under magnetic stirring. Supernatant was stirred overnight at 4°C. Precipitated proteins and particles were removed by centrifugation (4°C, 10.000 rpm, 30 min). The pellet was resuspended in 40% ice-cold acetone and again centrifuged. Both 40% acetone supernatant fractions were pooled. Thereafter the supernatant was brought to 60-80% acetone (v/v) and stirred overnight. Precipitated proteins were collected by centrifugation. The pellet was resuspended in 60-80% acetone and centrifuged again. The pellet was dissolved in an appropriate amount of a 5 mM acetic acid/ ammonium hydroxide buffer at pH 3.0 (buffer A) to a protein concentration of 20-50 g/l.

Ammonium sulphate precipitation

[0091] Polysaccharides were subsequently removed by precipitation of the gelatin / collagen at 60% saturation of ammonium sulphate, where the polysaccharides remained in solution. Ammonium sulphate was slowly added to 60% saturation at 4°C. After 60 min stirring the sample was centrifuged (30 min, 4°C, 10.000 rpm). The pellet was resuspended in 60% ammonium sulphate and again centrifuged. If more than 1 % (w/w) polysaccharides or sugars remained present, the complete ammonium sulphate precipitation procedure described above was repeated after complete redissolution of the gelatin / collagen in the absence of ammonium sulphate. Finally, the pellet was dissolved in deionized water or in buffer A to a protein concentration of 20-50 g/l. The pH of the sample was adjusted to 3.0. The sample was desalted by dialysis against buffer A, which was refreshed every 4 hours. Dialysis membranes of regenerated cellulose (Spectra Por®, from Spektrum) were used with a molecular weight cut-off of 8 kD. The dialysis was stopped after 2-7 days at the moment that the conductivity of the sample was judged to be sufficiently low (typically 20-150  $\mu\text{S}\cdot\text{cm}^{-1}$  above background). Conductivity was measured with a digital conductivity meter (Radiometer), calibrated with 1 mM and 10 mM KCl solutions (140 and 1400  $\mu\text{S}\cdot\text{cm}^{-1}$ , respectively). As an alternative to dialysis, ultrafiltration and diafiltration were used to desalt the samples and (optionally) to concentrate them. Where applicable, the product was subsequently pre-dried (optional) by precipitation with high concentrations of acetone and evaporation of the acetone, and finally freeze-dried.

## II. Purification by cation-exchange chromatography

[0092] The cation-exchange resin was SP Sepharose XL (Pharmacia Biotech), but other suitable resins could also be used. The purification was carried out at several scales. Thus, 25 mL bed in a XK16 column (Pharmacia) was used. Runs were performed with a FPLC (Pharmacia). Bed height was 12.5 cm. Flow rates were typically 1 ml/min. At an intermediate scale, a 100 mL bed was used, runs being controlled by an Äkta Explorer integrated pump/processor/multiple valve/multiple detector unit (Pharmacia). On pilot scale a 2 liter bed in an Index 140/200 column was used. Bed height was at least 13 cm. Runs were performed with the ÄKTA explorer pump-processor unit (Pharmacia) or other pump systems. Flow rates were 50-100 ml/min, or higher. As an example, the following buffer system and elution conditions were used. Buffer X was a 5 mM citric acid buffer at pH 3.2, buffer Y a 5 mM citric acid buffer with 1 M NaCl at pH 3.0. The column was equilibrated with 2-5 bed volumes of buffer X. The protein of interest was eluted with a linear gradient of 0-0.5 M NaCl in 5-10 column volumes. The main band of collagen type III eluted at 50-100 mM NaCl. The main band of collagen type I eluted at 70 mM NaCl, the other bands between 30-150 mM NaCl, in agreement with their theoretical isoelectric points. The column was cleaned with 1 bed volume of buffer Y. On a pilot scale the pooled fractions were desalted and concentrated, e.g. by addition of 80% acetone, and subsequently freeze-dried.

## Characterization of the gelatin / collagen product

[0093] The amino acid composition was determined after complete HCl-mediated hydrolysis of the peptide bonds at very low pH and elevated temperature, followed by derivatisation of the amino acids with a fluorophore, and HPLC.

[0094] The percentage Gly expected from pure collagen is 33%. This offers a means of estimating the purity of produced recombinant gelatins. In order to correct for the percentage of Gly in endogenously secreted proteins of *Pichia pastoris*, amino acid composition analysis was performed on fermentation supernatant of a Mut<sup>+</sup> transformant of pPIC9. The percentage Gly found was 9%. The purity of a sample can now be estimated by the formula:

$$(\% \text{Gly} - 9) / (33 - 9) = (\% \text{Gly} - 9) / 24.$$

[0095] After dissolution of samples in MilliQ water, the following assays were performed.

[0096] The protein content was determined by the BCA assay from Pierce, using gelatin from Merck as a reference.

[0097] The protein Mw distribution was determined by SDS-PAGE.

[0098] The sugar content was determined by a phenol-based assay. 200 µL samples were mixed with 200 µL 5 % (w/w) phenol. After thorough mixing using a Vortex mixer, 1 mL of concentrated sulphuric acid was added. After mixing, the samples were incubated for 10 min at room temperature and, subsequently, for 20 min. at 30 °C. After cooling, the light absorption of the samples at 485 nm was determined. Starch, glucose and sucrose were used to prepare calibration curves.

[0099] The DNA content was determined by mixing aliquots of diluted SYBR® Green I nucleic acid "gel" stain (10.000 x conc. in DMSO) from Molecular Probes with our samples. After thorough spectral analysis, the excitation wavelength was chosen to be 490 nm, and the emission wavelength 523 nm. The calibration was by subsequent addition of known amounts of DNA to this same mixture, as internal standards. Thus, a calibration curve was constructed. Furthermore, it was checked that subsequent addition of DNA-degrading enzyme resulted in complete break down of the fluorescent signal.

[0100] A quantitative indication of the RNA plus DNA-content was subsequently obtained by using SYBR® Green II "RNA gel stain", instead of SYBR® Green I. After thorough spectral analysis, the excitation wavelength was chosen to be 490 nm, and the emission wavelength 514 nm. Calibration was by subsequent addition of known amount of RNA. The resulting value was denoted the "RNA" content of the sample. In the absence of DNA, it corresponded to the true RNA content. When present, the DNA-associated fluorescence may have biased the RNA values, although a final addition of RNase was used to discern the DNA- and RNA-derived contributions to the fluorescence.

[0101] The conductivity of the samples was measured with a digital Radiometer conductivity meter, checking that 1 and 10 mM KCL solutions in MilliQ water gave readings of 140 and 1400 µS.cm<sup>-1</sup>, respectively.

#### Data on purity of some gelatin batches produced in accordance with the invention (examples)

##### GATO4a (co13a1)

##### [0102]

- about 2.4 gram
- purification:
  - micro filtration, precipitation (1x acetone fractionation (40 % / 80 %), 1x (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), dialysis against 5 mM CH<sub>3</sub>COOH/CH<sub>3</sub>COO<sup>-</sup> -buffer keeping the sample below pH 4 (initial pH about 3.5; buffer prepared by dilution from 500 mM acetic acid adjusted to pH 3.0 with 25 % NH<sub>4</sub>OH), lyophilization
- DNA: < 1 ppm (w/w)
- RNA: 12.7 ppm (w/w)
- total sugars: 4.5 % (w/w)
- gelatin was not degraded during purification (SDS-PAGE: Figure 14)

##### GATO4b (co13a1, further purified than GATO4a)

##### [0103]

- about 1 gram
- purification:
  - further purified from GATO4a by repeated (2x additional) ammonium sulphate precipitation followed by dialysis against 5 mM CH<sub>3</sub>COOH/CH<sub>3</sub>COO<sup>-</sup> - buffer keeping the sample below pH 4 (initial pH about 3.5; buffer prepared by dilution from 500 mM acetic acid adjusted to pH 3.0 with 25 % NH<sub>4</sub>O-H), lyophilization
- DNA: 0.56 ppm (w/w)
- RNA: 3.2 ppm (w/w)

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- total sugars: 0.94 % (w/w)
- gelatin was not degraded during purification.
- specific conductance after dialysis about  $180 \mu\text{Scm}^{-1}$  at 10 g gelatin/L (specific conductance of buffer about  $100 \mu\text{Scm}^{-1}$ )
- 5 - specific conductance after lyophilization and dissolving a sample:  $180 \mu\text{Scm}^{-1}$  at 10 g gelatin/L,  $100 \mu\text{Scm}^{-1}$  at 5 g gelatin/L

### GATO5 (col1a1-1)

#### 10 [0104]

- about 0.9 gram
- purification:  
15 micro filtration, precipitation (1x acetone fractionation (40 % / 80%), 1x  $(\text{NH}_4)_2\text{SO}_4$ ), dialysis against 5 mM  $\text{CH}_3\text{COOH}/\text{CH}_3\text{COO}^-$ -buffer keeping the sample below pH 4 (initial pH about 3.5; buffer prepared by dilution from 500 mM acetic acid adjusted to pH 3.0 with 25 %  $\text{NH}_4\text{OH}$ ), lyophilization
- DNA: < 1 ppm (w/w)
- RNA: 87 ppm (w/w)
- total sugars: 4.5 % (w/w)
- 20 - gelatin was not degraded during purification (SDS-PAGE: Figure 15)

### GATO6 (col3a1 purified by expanded bed cation exchange chromatography)

#### 25 [0105]

- about 50 mg
- purification:  
30 expanded bed cation exchange chromatography at pH 3-3.5 (SP-Sepharose-XL "Streamline" resin from Pharmacia Biotech; sugar content after sub-optimal washing of the column and elution at 0.3 M NaCl: 1.8 % (w/w)), further removal of sugar by a single  $(\text{NH}_4)_2\text{SO}_4$ -precipitation, followed by dialysis against 5 mM  $\text{NH}_4^+ / \text{CH}_3\text{COO}^-$ -buffer (pH about 3.5), lyophilization
- DNA : < 1 ppm(w/w, already before  $(\text{NH}_4)_2\text{SO}_4$ -precipitation and dialysis)
- RNA: < ppm (w/w, already before  $(\text{NH}_4)_2\text{SO}_4$ -precipitation and dialysis)
- total sugars: 1.1 %
- 35 - specific conductance after dialysis about  $94 \mu\text{Scm}^{-1}$  at 0.55 g gelatin/L (specific conductance of buffer: about  $100 \mu\text{Scm}^{-1}$ ).
- gelatin was not degraded during purification

### GATO7 (col1a1-2)

40

#### [0106]

- 400 mg
- purification:  
45 micro filtration, precipitation (1x acetone fractionation (40 % / 71.5 %), 3x  $(\text{NH}_4)_2\text{SO}_4$ ), pre-desalting by acetone precipitations: 1x 71.5 %, 1x 80 %, dialysis against MilliQ water, lyophilization
- DNA: 0,79 ppm
- RNA: 9,5 ppm
- total sugars: 0.7 % (w/w)
- 50 - specific conductance after dialysis about  $15.5 \mu\text{Scm}^{-1}$  at 4 g gelatin/L
- gelatin was not degraded during purification

### GATO8 (col3a1)

#### 55 [0107]

- about 6 g
- purification:

microfiltration, dilution, cation exchange chromatography in a 2,1 litre bed SP Sepharose-XL from Pharmacia Biotech equilibrated with 20 mM citrate, pH 3,5 and elution at 0.15 M NaCl in the same buffer over a gradient of 0-1 M NaCl, concentration, partial desalination with 80% acetone, centrifugation, resolubilisation in MilliQ water, dialysis against MilliQ water and lyophilisation

- DNA : 1,55 ppm(w/w)
- RNA: 10,9 ppm (w/w)
- total sugars: 1.2 % (w/w)
- specific conductance after dialysis about 90  $\mu\text{Scm}^{-1}$  at 7,5 g gelatin/L
- gelatin was not degraded during purification

Figure 16 shows the result of purification.

#### **GATO9 (col1a1-1)**

##### **[0108]**

- 1,7 g
- purification: see GATO8 with one difference i.e. elution from the cationic exchanger in a 1 salt step at 0,75 M NaCl.
- DNA: <1 ppm (w/w)
- RNA: 1,3 ppm (w/w)
- total sugars: 2,2 % (w/w)
- specific conductance after dialysis about 70  $\mu\text{Scm}^{-1}$  at 12 g gelatin/L
- gelatin was not degraded during purification

#### **GATO10 (col1a1-2) wherein both MGPR sequences have been changed to RGPM**

##### **[0109]**

- 6 g
- purification: see GATO8
- DNA: 0,04 ppm (w/w)
- RNA: 2 ppm (w/w)
- total sugars: 2 % (w/w)
- gelatin was not degraded during purification
- N terminal amino acid sequence was as expected with Glu-Ala as N terminal extension due to incomplete removal of the propeptide.

The results are shown in Figure 18.

**[0110]** In conclusion the amino acid composition of all samples matched the theoretical composition. The contamination with foreign protein was very low. On a glycine basis GATO4-GATO8 have less than 1% foreign protein as contaminant. GATO9 and GATO10 have less than 5%.

Description to the figures

##### **[0111]**

Figure 1: vector pCOL3A1

Figure 2: PCR primers for pCOL3A1 construct control

Figure 3: The expected COL3A1 sequence. The N terminal Y is derived from the pPIC 9 vector. The rest of the sequence is derived from COL3A1 of the rat. The underlined sequences correspond to the N terminal sequences obtained for COL3A1 fragments.

Figure 4: vector pCOL3A1K.

Figure 5: Oligo sequences for cloning of COL1A1. The bottom sequence is the adaptor after annealing.

Figure 6: Cloning strategy

Figure 7: vector pCOL1A1-1

Figure 8: The expected COL1A1-1 sequence. The singly underlined sequences correspond to the N terminal sequences obtained for COL1A1 fragments. The double underlined sequences share this sequence. Both fragment are extended at the N terminus by EA.



Figure 9: vector pCOL1A1-2

Figure 10: Expected COL1A1-2 sequence

Figure 11 vector pCOL1A1-3

Figure 12 Expected COL1A1-3\* sequence

Figure 13: MGPR sequence in the expected COL1A1-2 sequence. The singly underlined sequences correspond to the N terminal sequences obtained for COL1A1-1 fragments. The double underlined sequences is the MPPR sequence.

Figure 14: SDS poly acryl amide gel electrophoresis of GATO4. The gel was stained with Coomassie Brilliant Blue. In the left most lane the molecular marker protein mix is visible. From top to bottom the bands correspond to molecular weights of 94,67,43,30,20 and 14,4 kD. The second and third lane from the left show GATO4 after purification.

Figure 15: SDS poly acryl amide gel electrophoresis of GATO5. The gel was stained with Coomassie Brilliant Blue. In the right most lane the molecular marker protein mix is visible. From top to bottom the bands correspond to molecular weights of 94,67,43,30,20 and 14,4 kD. The second and third lane from the right show GATO5 after purification.

Figure 16 SDS poly acryl amide gel electrophoresis of expression product col1A1-2. The gel was stained with Coomassie Brilliant Blue. In the left most lane the molecular marker protein mix is visible. From top to bottom the bands correspond to molecular weights of 94, 67, 43, 30, 20,1 and 14,4 kD.

Figure 17 SDS poly acryl amide gel electrophoresis of expression product col1A1-1 in which MGPR sequences have been mutated to RGPM. The gel was stained with Coomassie Brilliant Blue. In the left most lane the molecular marker protein mix is visible. From top to bottom the bands correspond to molecular weights of 94, 67, 43, 30, 20,1 and 14,4 kD.

Figure 18 SDS poly acryl amide gel electrophoresis of expression product col1A1-2 in which MGPR sequences have been mutated to RGPM. The gel was stained with Coomassie Brilliant Blue. In the left most lane the molecular marker protein mix is visible. From top to bottom the bands correspond to molecular weights of 94, 67, 43, 30, 20,1 and 14,4 kD.

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#### Example 2 (control)

Preparation of silver bromide crystals with conventional regular type gelatin.

**[0113] Nucleation:** At a temperature of 35°C in a reaction vessel containing 2.1 g/l regular gelatin (a standard deionised IAG bone gelatin from PB Gelatins, Tessenderlo, Belgium) and 7.3 mM potassium bromide the pH is adjusted to a value of 5.5 by sodium hydroxide or sulphuric acid. By single jet addition an aqueous solution of 144 mM silver nitrate is added at a constant rate in a period of 10 sec while vigorously stirring. After addition of the silver nitrate the gelatin concentration in the reaction mixture has become 2.0 g/l and the bromide concentration 1 mM.

**[0114] Ripening:** After nucleation the content of the vessel is transferred to a ripening vessel, where the temperature is increased gradually to a value of 75°C and the bromide concentration is increased to 15 mmol/l by adding a 3.4 M potassium bromide solution. The ripening is continued for 56 minutes after which a standard deionised gelatin is added up to a concentration of 5 g/l; after 58 minutes the Ostwald ripening is strongly reduced by adding a solution of methyl phenyl tetrazole and cooling to room temperature. A sample of the prepared emulsion was analyzed by direct transmission electron microscopy as well as by replica thereof.

**[0115] Result:** As can be seen in table II very low % of tabular grains is formed.

#### Example 3 (control)

Preparation of silver bromide crystals with conventional hydrolysed gelatin.

**[0116] Nucleation:** The nucleation is performed applying the same conditions (also pH=5.5) as in example 2 except that the gelatin in the reaction mixture is replaced by a conventional hydrolysed gelatin sample (also deionised and supplied by Nitta Gelatin in Japan).

**[0117] Ripening:** The ripening is done according the same procedure as is used in example 2.

**[0118] Result:** A medium % tabular grains of ca 40% is shown in table II.

#### Example 4 (control)

Preparation of silver bromide crystals with oxidized gelatin.

**[0119] Nucleation:** The nucleation is performed applying the same conditions (also pH=5.5) as in example 1 except that the gelatin in the reaction mixture is replaced by a conventional oxidized gelatin sample (supplied by PB Gelatins Tessenderlo in Belgium).

**[0120] Ripening:** The ripening is done according the same procedure as is used in example 2.

**[0121] Result:** A high % tabular grains of 70% with an average aspect ratio of 5:1 is shown in table II for the oxidized gelatins at pH 5.5. The better result than with the hydrolysed and regular gelatin is to be explained due to the lower methionine content of this gelatin (11 µmol/gram gelatin vs. 50-60 µmol/gram for the conventional gelatins).

#### Example 5 (this invention)

Preparation of silver bromide crystals with invented native recombinant gelatins.

**[0122] Nucleation:** The nucleation is performed applying the same conditions (also pH=5.5) as in example 2 except that the gelatin in the reaction mixture is replaced by the invented native COL3A1 gelatin sample.

**[0123] Ripening:** The ripening is done according to the same procedure as is used in example 2.

**[0124] Result:** A high % tabular grains of more than 85% with an average aspect ratio of 5:1 is shown in table II.

**Example 6 (control)**

Preparation of silver bromide crystals with conventional regular gelatin at different pH (Standard deionised IAG bone gelatin from PB Gelatins, Tessenderlo in Belgium).

[0125] Nucleation: the nucleation is performed applying a pH=7 condition while the other conditions remained the same as in example 2.

[0126] Ripening: The ripening is done according to the same procedure as is used in example 2 except the pH remained the same i.e. pH=7 as during the nucleation.

[0127] Result: No tabular grains of aspect ratio larger than 5 resulted as is shown in table II for conventional commercial gelatin.

**Example 7 (control)**

Preparation of silver bromide crystals with conventional hydrolysed gelatins at a different pH (Nitta Gelatins in Japan).

[0128] Nucleation: The nucleation is performed applying a different pH=7 condition while the other conditions remained the same as in example 2.

[0129] Ripening: the ripening is done according to the same procedure as is used in example 2 except the pH remained the same at pH=7 as during the nucleation.

[0130] Result: A very low % tabular grains around 5% resulted as is shown in table II.

**Example 8 (this invention)**

Preparation of silver bromide crystals with invented native recombinant gelatin at a different pH.

[0131] Nucleation: The nucleation is performed applying a different pH condition i.e. pH=7 while the other conditions remained the same as in example 4.

[0132] Ripening: The ripening is done according to the same procedure as is used in example 4 except the pH remained the same i.e. at pH=7 as during the nucleation.

[0133] Result: A very high % tabular grains ca 80% is surprisingly found at this condition which is clearly higher than the state-of-the-art gelatins as is shown in table II.

**Example 9**

Relation between binding strength and tabular grain morphology.

[0134] 45 mg gelatin is accurately weighed and 15 g 0.1M phosphate buffer pH=7.00 containing 0.1M potassium nitrate, is added. The solution is placed in a waterbath at 45°C for 15 minutes. The solution is cooled to room temperature (23°C).

10 ml of this pH 7.0 phosphate buffer solution (containing gelatin) is mixed at 23°C with 100 µl 0.5 mM silver nitrate. The potential of this solution entitled as "vAg" is measured using an Ag electrode (Orion model 97-81) against an Ag/AgCl reference double junction electrode (Orion model 90-02). The same buffer solution without gelatin is also mixed with the silver nitrate solution and the potential "vAg" measured by the same method. The difference between the two measured potentials is calculated and expressed as "delta vAg" being the binding affinity of gelatin for the Ag-ion. Table II below contains the tested peptizers, the % tabular grains and the gelatin binding affinities "delta vAg" for pH 5.5 and pH 7 in which the criteria for tabularity has been defined at aspect ratio > 5:

Type gelatin	Bind. strength "delta vAg" (mV)	% tabular at nucleation/ ripening pH=5.5	% tabular at nucleation/ ripening pH=7.0
oxidized	55.4	73	-*
hydrolysed	74.4	42	5
regular	78.5	1	0
nat.rec COL3	69.5	87	79

\*not measured

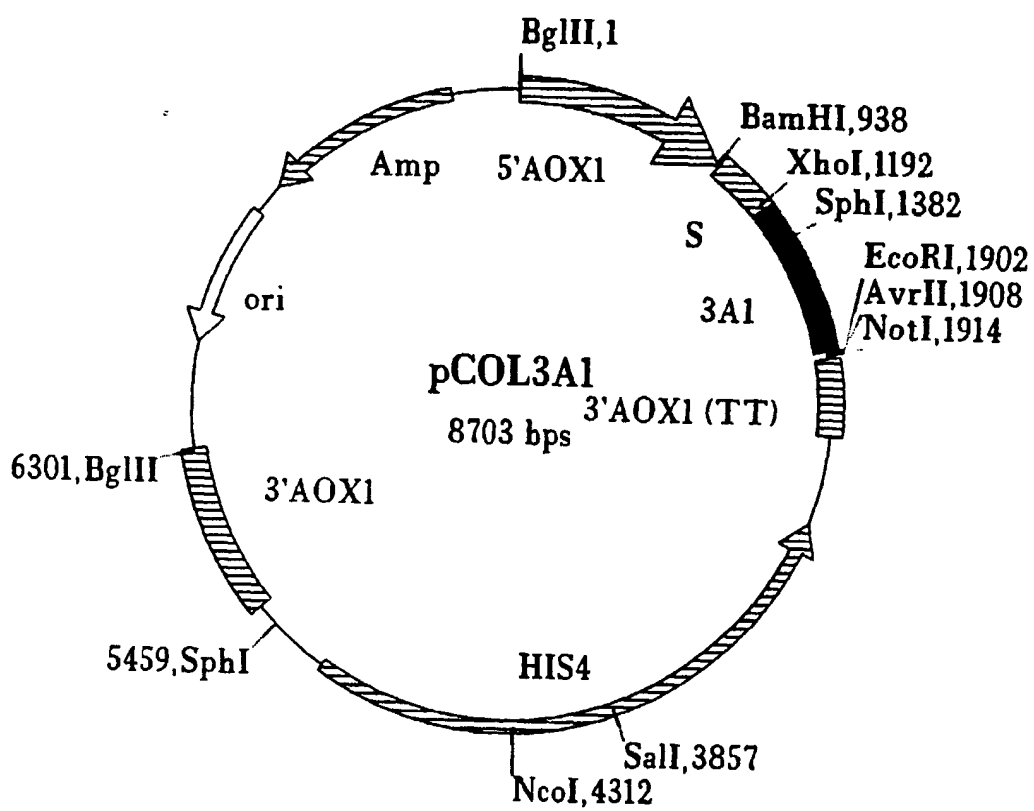
## Claims

1. A tabular silver halide emulsion wherein the tabular grains account for more than 75% of the total grain projected area said emulsion comprising silver halide grains nucleated in the presence of a nucleation peptizer and thereafter grown in the presence of a growth peptizer, wherein at least one of the peptizers is substantially pure collagen like materials prepared by genetic engineering of native collagen encoding nucleic acid, said peptizer having an amino acid sequence comprising more than 4 different amino acids.
2. An emulsion according to claim 1, said peptizer having an amino acid sequence comprising more than 4, even more than 10 amino acids that are different.
3. An emulsion according to claim 1 or 2 wherein the amino acid sequence of the peptizer exhibits more than 40% homology with native collagen, preferably more than 50%.
4. An emulsion according to any of the preceding claims said peptizer having an amino acid sequence equivalent to that occurring in nature for collagen, wherein equivalent implies amino acid identity of at least 80%, preferably at least 90%, most preferably with an amino acid sequence the same as occurs in nature.
5. An emulsion according to any of the preceding claims, wherein the peptizer has an amino acid sequence substantially the same as occurs in nature, wherein substantially implies mutation of less than 5 amino acids, preferably less than 3.
6. An emulsion according to any of the preceding claims, wherein the peptizer has an amino acid sequence substantially the same as occurs in nature for collagen type I, II or III.
7. An emulsion according to any of the preceding claims, wherein the peptizer has an amino acid sequence equivalent to the amino acid sequence occurring in nature for collagen type I, II or III.
8. An emulsion according to any of the preceding claims, wherein the peptizer has an amino acid sequence the same as occurs in nature for collagen type I, II or III.
9. An emulsion according to any of the preceding claims, wherein the collagen type I comprises or has the amino acid sequence of figure 8, 10 or 12 or Genbank accession number U08020.
10. An emulsion according to any of claims 1-7, wherein the collagen type III comprises or has the amino acid sequence of figure 3 or EMBL accession number X70-369.
11. An emulsion according to any preceding claim, said peptizer having an amino acid sequence substantially the same as occurs in nature for collagen type I, the peptizer being free of a sequence [MLIV]XXR and preferably free of MGPR.
12. An emulsion according to any of the preceding claims, wherein the peptizer is a fragment of defined length and composition derived from a native collagen encoding sequence, said fragment comprising the GXY motif characteristic of collagen, said length being such that the fragment weight on amino acid basis is at least 2,5kDa.
13. An emulsion according to any of the preceding claims, wherein the peptizer is free of hydroxyproline.
14. An emulsion according to any of the preceding claims, wherein the peptizer is not deaminated.
15. An emulsion according to any of the preceding claims, wherein the peptizer has an isoelectric point of 7-10.
16. An emulsion according to any of the preceding claims, wherein the peptizer has a weight on amino acid basis of 2,5-100kDa.
17. An emulsion according to any of the preceding claims, said peptizer being free of procollagen and telopeptides.
18. An emulsion according to any of the preceding claims, said peptizer being free of helix structure.

19. An emulsion according to any of the preceding claims, said peptizer being free of cysteine.
20. An emulsion according to any of the preceding claims, said peptizer comprising (oxidated) reducing amino acids to a degree that reducing amino acids methionine and histidine are present at a level equivalent to a reducing strength of between 0,1-200, preferably between 0,1-120 micromoles of methionine per gram of said peptizer.
21. An emulsion according to any of the preceding claims, said peptizer comprising (oxidated) reducing amino acids to a degree that reducing amino acids are present at a level equivalent to a reducing strength of between 0,1-80 micromoles of methionine per gram of said peptizer.
22. An emulsion according to any of the preceding claims, said peptizer comprising (oxidated) reducing amino acids to a degree that reducing amino acids are present at a level equivalent to a reducing strength of between 30-80 micromoles of methionine per gram of said peptizer.
23. An emulsion according to any of the preceding claims, said peptizer having a binding strength for silver higher than 50 mV,
24. An emulsion according to any of the preceding claims, said peptizer having a binding strength for silver below 100 mV,
25. An emulsion according to any of the preceding claims, said peptizer having a binding strength for silver between 50-100 mV.
26. An emulsion according to any of the preceding claims, wherein the silver halide grains exhibit an average aspect ratio higher than 5.
27. An emulsion according to any of the preceding claims, wherein the peptizer is stable vis a vis silver halide tabular grain formation at a pH between 4-8.
28. An emulsion according to any of the preceding claims comprising tabular grains at a level more than 90%, preferably more than 95%
29. An emulsion according to any of the preceding claims wherein the peptizer is of a homo disperse nature.
30. An emulsion according to any of the preceding claims wherein the peptizer is present in substantially pure form i. e. substantially free of nucleic acid, polysaccharides and other protein.
31. An emulsion according to any of the preceding claims, said emulsion being suitable for photographic application and comprising further compounds acceptable for such application, e.g a second clearly defined amount of growth peptizer.
32. A process of preparing tabular silver halide emulsion wherein the tabular grains account for more than 75% of the total grain projected area said process comprising nucleation of silver halide grains in the presence of nucleation peptizer and thereafter growing said silver halide grains in the presence of growth peptizer, wherein both peptizers are present in a defined amount and at least one peptizer is collagen like material prepared by genetic engineering of native collagen encoding nucleic acid, said peptizers having an amino acid sequence comprising more than 4 different amino acids.
33. A process for preparing an emulsion according to claim 32 comprising addition of the peptizer in the nucleation step and/or during the grain growing step, said peptizer being selected from any of the embodiments disclosed in any of claims 1-31, 51 or 52.
34. A process for preparing an emulsion according to claim 32 or 33 comprising addition of the peptizer both in the nucleation step and during the grain growing step.
35. A process for preparing an emulsion according to any of claims 32-34 comprising addition of the same peptizer in the nucleation step and during the grain growing step.

36. A process of photographic element production comprising application of an emulsion according to any of claims 1-31 or obtainable from a process according to any of claims 32-35 for obtaining a silver halide emulsion to be applied in a manner known per se for photographic element production with at least one silver halide emulsion layer, wherein the silver halide crystals of said layer have an aspect ratio of 5 or more.
37. A process according to claim 36, said photographic element being a material sensitive to light, laser or x-ray radiation, said element e.g. being selected from black and white reversal film, black and white negative film, colour negative film, colour reversal film, film in which the sensitive photographic components are digitally scanned, black and white reversal paper, black and white paper, colour paper, reversal colour paper, paper in which the sensitive photographic components are sensitized by laser radiation out of a digital database.
38. Photographic element obtained according to the process according to claim 36 or 37.
39. A process of producing recombinant collagen like polypeptide comprising expression of a collagen like polypeptide encoding nucleic acid sequence by a microorganism to a degree exceeding 0.95 gram /liter, said recombinant collagen being free of helix structure and preferably the expression occurring in a microorganism other than E. coli or Saccharomyces cerevisiae.
40. A process according to claim 39 wherein the expression exceeds 3 gram /liter.
41. A process according to claim 39 or 40 wherein the recombinant collagen like peptide is selected from any of the embodiments disclosed in any of claims 1-31, 51 or 52.
42. A process according to any of claims 39-41 wherein the microorganism is capable of secreting expression product.
43. A process according to any of claims 39-42 wherein the microorganism is a fungal cell preferably a yeast cell.
44. A process according to any of claims 39-43 wherein the microorganism is free of active post translational processing mechanism for processing collagen like sequences to fibrils.
45. A process according to any of claims 39-44 wherein the microorganism is free of active post translational processing mechanism for processing collagen like sequences to triple helices.
46. A process according to any of claims 39-45 wherein the nucleic acid sequence to be expressed is free of procollagen and telopeptide encoding sequences.
47. A process according to any of claims 39-46 wherein the microorganism is selected from the group consisting of Hansenula, Trichoderma, Aspergillus, and preferably Pichia pastoris.
48. A process according to any of the claims 39-47 wherein the recombinant collagen has an amino acid sequence free of protease cleavage sites of any protease active in the expression host cell.
49. A process according to any of claims 39-48 wherein the expression product is isolated and purified until it is substantially free of other protein, polysaccharide and nucleic acid.
50. A process according to any of claims 39-49 wherein the expression product is isolated and purified to at least the following degree: content nucleic acid less than 100 ppm, content polysaccharide less than 5%, suitably RNA content less than 10 ppm, suitably DNA content less than 1 ppm most preferred.
51. A substantially pure collagen like material prepared by genetic engineering of native collagen encoding nucleic acid, said peptide having an amino acid sequence exhibiting more than 40% homology with native collagen and comprising more than 4 different amino acid types.
52. A recombinant collagen compound as defined in any of the claims 1-31.

**Fig 1**



*Fig 2*

5'AOX1: 5'-GACTGGTTCCAATTGACAAGC-3'

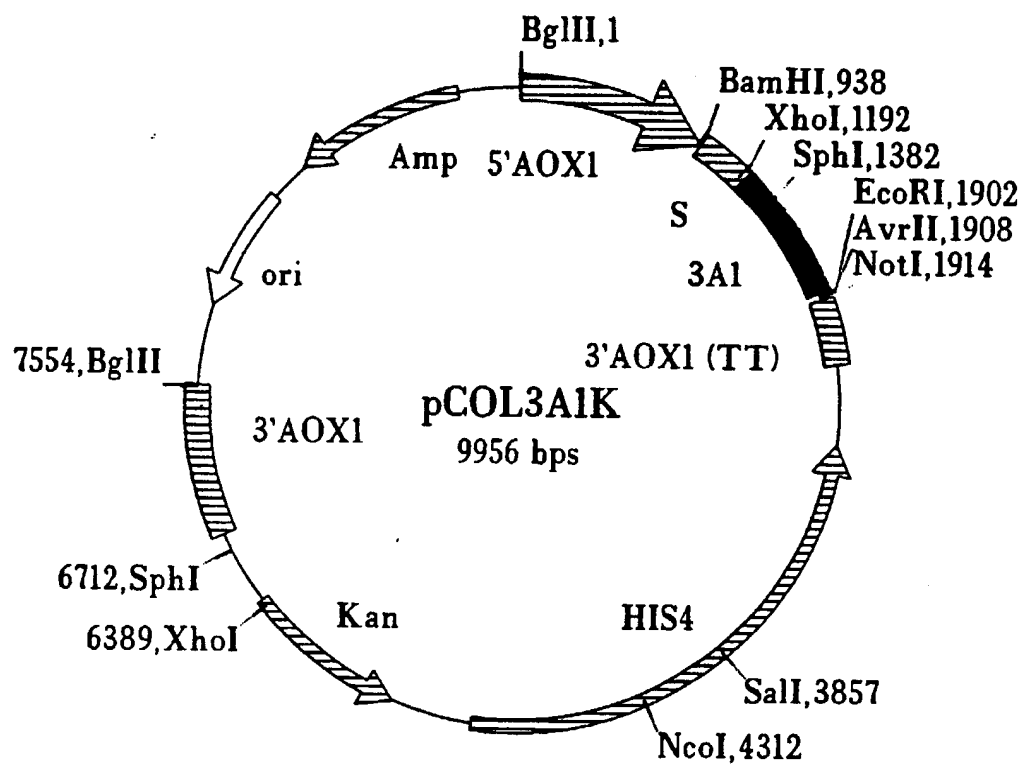
3'AOX1: 5'-GCAAATGGCATTCTGACATCC-3'

 $\alpha$ MF: 5'-TACTATTGCCAGCATTGCTGC-3'*Fig 3*

1 YGNSGSPGNP GVAGPKGDAG QPGEKGPPGA QGPPGSPGPL GIAGLTGARG LAGPPGMPPG  
 61 RGSPGPQGIK GESGKPGASG HNGERGPPGP QGLPGQPGTA GEPGRDGNPG SDGQPGRDGS  
 121 PGGKGDRGEN GSPGAPGAPG HPGPPGPVGP SGKNGDRGET GPAGPSGAPG PAGARGAPGP  
 181 QGPRGDKGET GERGSNGIKG HRGFPGNPGP PGSPGAAGHQ GAVGSPGP



**Fig 4**

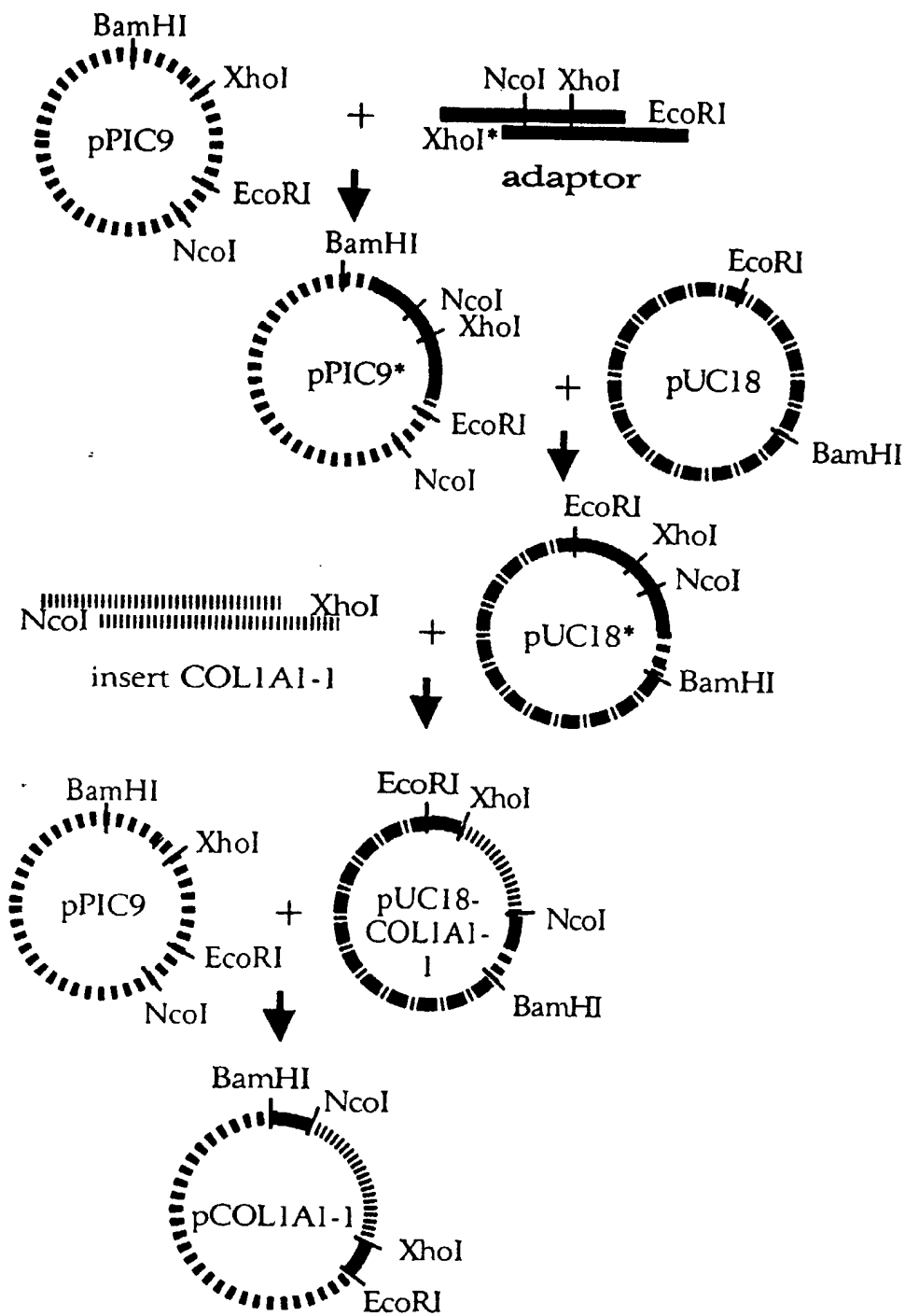


*Fig 5*

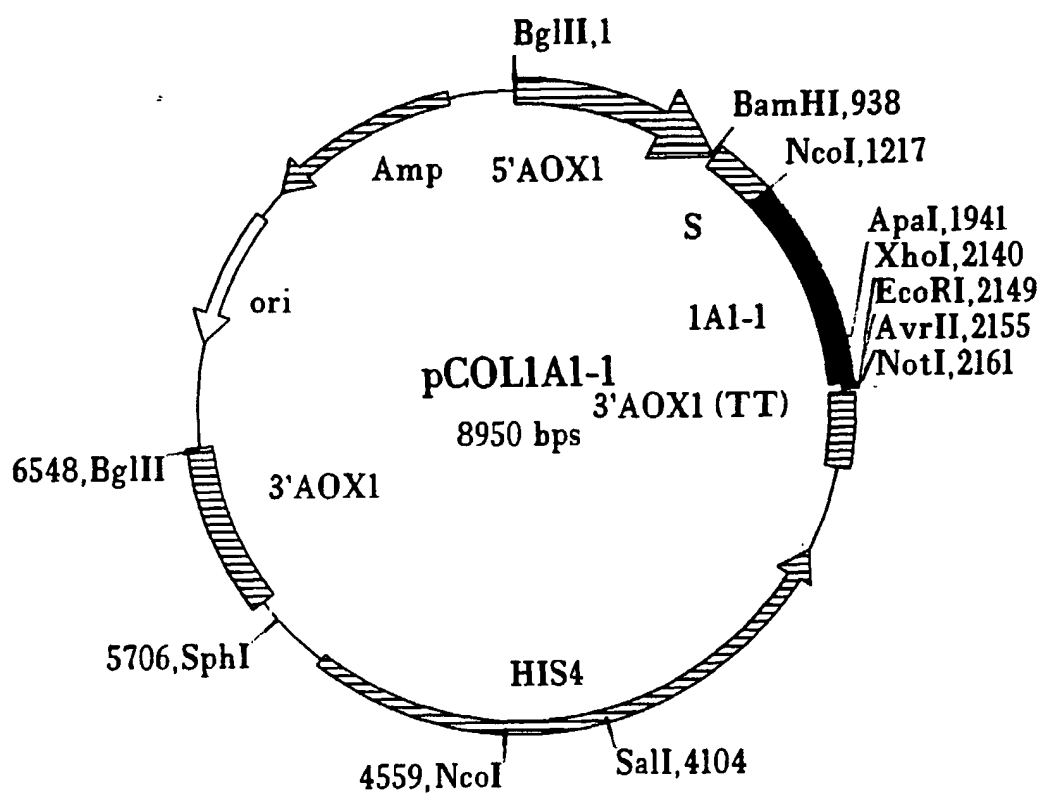
C1A1-FW : 5'-CTTCCCAGATGTCCTATGGCTATGATG-3'  
 C1A1-RV1 : 5'-CCGCTCGAGGCGCTCGCCAGGAGGTCCAGGCAG-3'  
 C1A1-RV2 : 5'-GCGCTCGAGGGGAGGACCAATGGGACCAGTCAG-3'  
 C1A1-RV3 : 5'-GCGCTCGAGGCCAGGAGAACCAAGGAGGACCCCTG-3'

N-X-FW : 5'-TCGAAAAGAGAGAGGCTGAAGCTCCCATGGGATAACTCGAGTAGG-3'  
 N-X-RV : 5'-AATTCCTACTCGAGTTATCCCATGGGAGCTTCAGCCTCTCTCTTT-3'

5'-TCGAAAAGAGAGAGGCTGAAGCTCCCATGGGATAACTCGAGTAGG-3'  
 3'-TTTCTCTCTCCGACTTCGAGGGTACCCCTATTGAGCTCATCCTTAA-5'

*Fig 6*

**Fig 7**



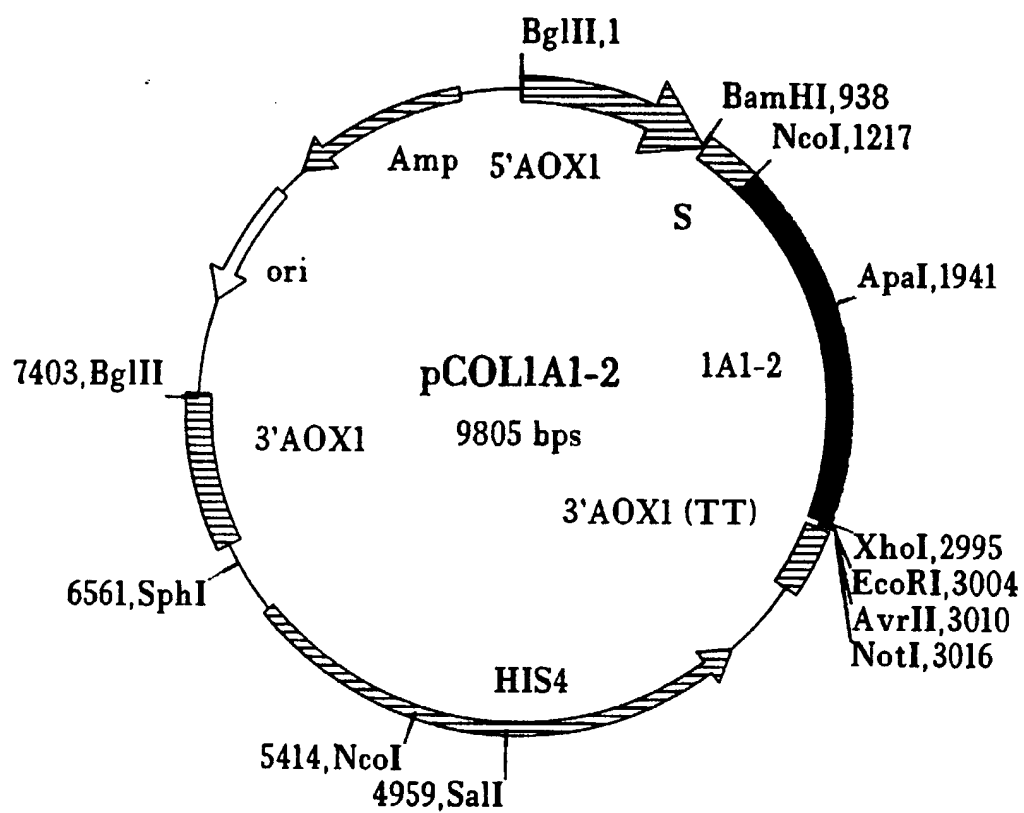
*Fig 8*

1 PMGPSGPRGL PGPPGAPGQ GFQGPPEPG EPGSGPMGP RGPPGPPGKN GDDGEAGKPG  
 61 RPERGPPGP QGARGLP<sub>GT</sub>A GLPGMKGHRG FSGLDGAKGD AGPAGPKGEP GSPGENGAPG  
 121 QMGPRGLPGE RGRPGPPGT<sub>A</sub> GARGNDGAVG AAGPPGPTGP TGPPGFPGAV GAKGEAGPQG  
 181 ARGSEGPOGV RGEPPGPPGA GAAGPAGNPG ADQPGAKGA NGAPGIAGAP GFPGARGPSG  
 241 PQGPSGPPGP KGNSGEPGAP GNKGD<sub>T</sub>GAKG EPGATGVQGP PGPAGEEGKR GARGEPPSG  
 301 LPGPPGERLE

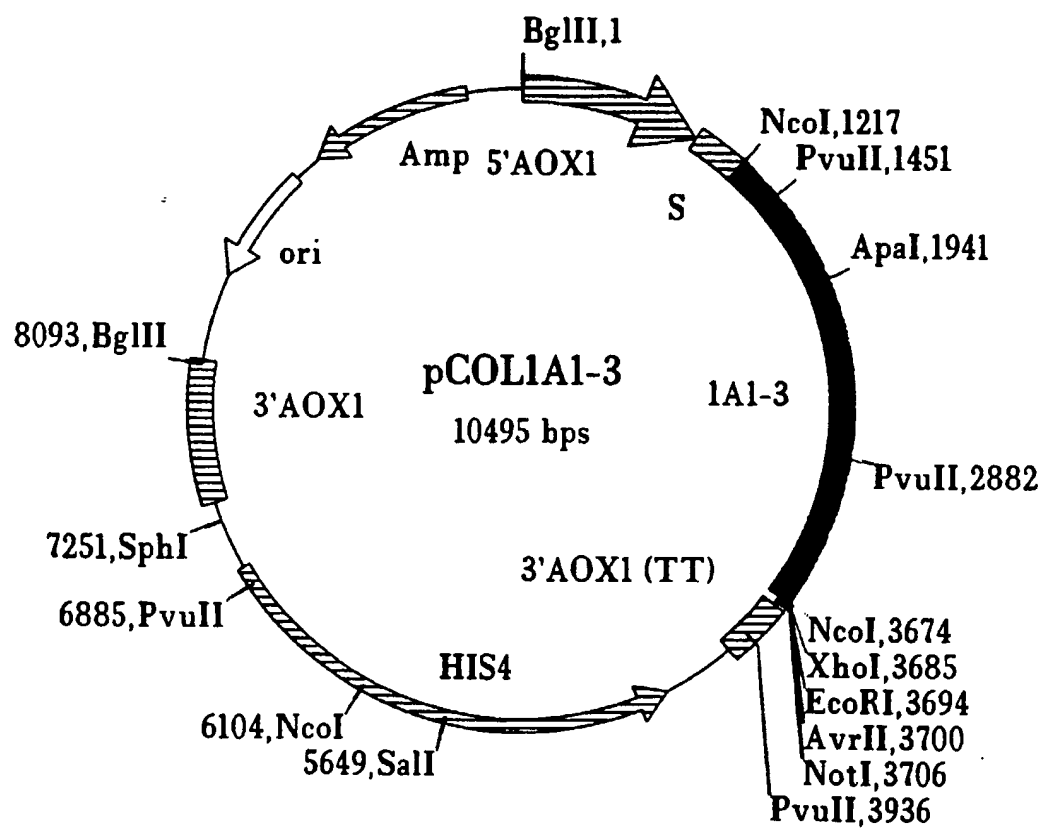
*Fig 10*

1 PMGPSGPRGL PGPPGAPGQ GFQGPPEPG EPGSGPMGP RGPPGPPGKN GDDGEAGKPG  
 61 RPERGPPGP QGARGLP<sub>GT</sub>A GLPGMKGHRG FSGLDGAKGD AGPAGPKGEP GSPGENGAPG  
 121 QMGPRGLPGE RGRPGPPGT<sub>A</sub> GARGNDGAVG AAGPPGPTGP TGPPGFPGAV GAKGEAGPQG  
 181 ARGSEGPOGV RGEPPGPPGA GAAGPAGNPG ADQPGAKGA NGAPGIAGAP GFPGARGPSG  
 241 PQGPSGPPGP KGNSGEPGAP GNKGD<sub>T</sub>GAKG EPGATGVQGP PGPAGEEGKR GARGEPPSG  
 301 LPGPPGERGG PGSRGFP<sub>GAD</sub> GVAGPKGPSG ERGAPGPAGP KGSPGEAGRP GEAGLP<sub>GAKG</sub>  
 361 LTGSPGSPGP DGKTGPPGPA GQDGRPPAG PPARGQAGV MGFP<sub>GKGT</sub>A GEPKAGERG  
 421 LPGPPGAVGP AGKDG<sub>EAG</sub>AQ GAPGAPGAG ERGEQGPAGS PGFQGLP<sub>GPA</sub> GPPGEAGKPG  
 481 EQGVPGDLGA PGPSGARGER GFPGERGVOG PPGPAGPRGN NGAPGNDGAK GDTGAPGAPG  
 541 SQAPGLQGM PGERGAAGLP GPKGDRGDAG PKGADGSPGK DGARGLT<sub>GPI</sub> GP<sub>P</sub>LE

**Fig 9**



**Fig 11**



*Fig 12*

1 PMGPSGPRGL PGPPGAPGQ GFQGPPEPG EPGSGPMGP RGPPGPPGKN GDDGEAGKPG  
 61 RPERGPPGP QGARGLPFTA GLPGMKHHRG FSLDGAKGD AGPAGPKGEP GSPGENGAPG  
 121 QMGPRGLPGE RGRPPPGTA GARGNDGAVG AAGPPGPTGP TGPPGFPGAV GAKGEAGPQG  
 181 ARGSEGPQGV RGEPPPGPA GAAGPAGNPG ADGQPGAKGA NGAPGIAGAP GFPGARGPSG  
 241 PQGPSGPPGP KGNSGEPGAP GNKGDGAKG EPGATGVQGP PGPAGEEGKR GARGEPPSG  
 301 LPGPPGERGG PGSRGFPAGD GVAGPKGPSG ERGAPGPAGP KGSPGEAGRP GEAGLPKAKG  
 361 LTGSPGSPGP DGKTGPPGPA GQDGRPPAG PPGARGQAGV MGFPKPGTA GEPKAGERG  
 421 LPGPPGAVGP AGKDGEAGAQ GAPGPAGPAG ERGEQGPAGS PGFQGLPGA GPPGEAGKPG  
 481 EQGVPGDLGA PGPSGARGER GFPGERGVQG PPGAGPRGN NGAPGNDGAK GDTGAPGAPG  
 541 SQGAPGLQGM PGERGAAGLP GPKGDRGDAG PKGADGSPGK DGARGLTGP I GPPGPAGAPG  
 601 DKGEAGPSGP PGPTGARGAP GDRGEAGPPG PAGFAGPPGA DQQPGAKGEP GDTGVKGDAG  
 661 PPGPAGPAGP PGPIGNVGAP GPKGPRGAAG PPGATGFPGA AGRVPPGPS GNAGPPGPPG  
 721 PVGKEGKGP RGETGPAGRP GEVGP GPPG PAGEKSPGA DGPAGSPGP GQGIAGQRG  
 781 VWLPGQRGE RGFPGLGPS GEPKQGPSG SSGERGPPG MG



Fig 13

1 PMGPSGRGL PGPPGAPGQ GFQPPGEPG EPGSGPMGP RGPPGPPGKN GDDGEAGKPG  
 61 RPSGERPPGP QGARGLPQT GLPGMKHGRG FSGLDGAAGD AGPAGPKGEP GSPGENGAPG  
 121 QMGPRLPGE RGRPGPPGTA GARGNDGAVG AAGPPGPTGP TGPPGFPGAV GAKGEAGPQG  
 181 ARGSEGPQGV RGEPPPGPA GAAGPAGNPG ADGQPGAKGA NGAPGIAGAP GFPGARGPSG  
 241 PQGPSGPPGP KNSGEPGAP GNKGDGAKG EPGATGVQGP PGPAGEEGKR GARGEPPSG  
 301 LPGPPGERGG PGSRGFPAGD GVAGPKGPSG ERGAPGPAGP KGSPGEAGRP GEAGLPGAKG  
 361 LTGSPGSPGP DGKTGPPGPA GQDGRPPGAG PPGARGQAGV MGFPGPKGTA GEPGKAGERG  
 421 LPGPPGAVGP AGKDGEGAGQ GAPGPAGPAG ERGEQGPAGS PGFQGLPGA GPPGEAGKPG  
 481 EQGVPGDLGA PGPSGARGER GFPGERGVQG PPGPAGPRGN NGAPGNDGAK GDTGAPGAPG  
 541 SQGAPGLQGM PGERGAAGLP GPKGDRGDAG PKGADGSPGK DGARLTGPI GPPLE